

# Mechanisms for amplified mediator release from colonic mast cells: Implications for interstitial inflammatory diseases

Kim E. Barrett

**Kim E. Barrett**, Division of Gastroenterology, Department of Medicine, University of California, San Diego, School of Medicine, San Diego, CA 92103, USA

**Supported by** the grants from the National Institutes of Health, USA (DK33491 and AT01180) and the Crohn's and Colitis Foundation of America

**Correspondence to:** Kim E. Barrett, Ph.D., UCSD Medical Center, 8414, 200 west Arbor Drive (for courier delivery, use CTF-A108, 210 Dickinson Street), San Diego, CA 92103-8414, USA. kbarrett@ucsd.edu

**Telephone:** +1-619-543 3726 **Fax:** +1-619-543 6969

**Received:** 2004-02-11 **Accepted:** 2004-02-14

Barrett KE. Mechanisms for amplified mediator release from colonic mast cells: Implications for interstitial inflammatory diseases. *World J Gastroenterol* 2004; 10(5): 617-619  
<http://www.wjgnet.com/1007-9327/10/617.asp>

The mast cell is an enigmatic cell type whose physiological function has preoccupied large numbers of investigators for decades<sup>[1]</sup>. Some have concluded that the absence of mast cells is incompatible with life, at least in humans, because no human conditions have been documented where these cells are absent from the body. On the other hand, mice harboring specific mutations in certain growth factors, or their receptors, that lead to apparently an almost total ablation of the mast cell lineage, are viable, although they do have several documented abnormalities and may exhibit altered inflammatory responses in a variety of tissues<sup>[2]</sup>. The viability of such animals may reflect redundancy in the murine system for specific mast cell functions, and/or that other cell types adapt to become repositories of characteristic mast cell mediators. But in any event, mast cells have long been considered to play specific roles in pathophysiology, particularly in disease states that are characterized by allergic inflammation<sup>[1-5]</sup>. In the setting of the gastrointestinal tract, release of mast cell mediators has been thought to contribute to tissue injury and inflammation, as well as alterations in epithelial and smooth muscle function, in conditions such as food allergy, systemic anaphylaxis, ulcer disease and inflammatory bowel diseases, as well as, more controversially, irritable bowel syndrome<sup>[3,5-13]</sup>. The spectrum of mast cell involvement has also been expanded by recognition that they can participate in biological events not classically related to allergic responses, such as innate immunity, the phagocytosis of bacteria and cross-talk with the peripheral and enteric nervous systems<sup>[14-16]</sup>.

Mast cells are activated classically by cross-linking of membrane IgE receptors<sup>[17]</sup>. *In vivo*, this occurs when a genetically susceptible individual mounts an IgE antibody response to a foreign protein that would be seen as innocuous by the immune system of those who are not allergic. The IgE antibodies bind with great avidity to the mast cell IgE receptors. Thus, because mast cells in the tissues are also long-lived, the allergic individual becomes chronically sensitized, with mast cell IgE receptors occupied by allergen-specific IgE, priming the cell to be activated by a subsequent exposure to the allergen. Binding of allergen to adjacent IgE molecules on such primed

cells results, in turn, in apposition of the IgE receptors, thereby initiating a signal transduction cascade, involving, among other steps, mobilization of intracellular calcium and activation of protein kinases, that leads ultimately to release of mast cell mediators<sup>[17,18]</sup>. The mediators that account for the biological effects of mast cell activation may be stored in cytoplasmic granules, such as histamine and a protease known as tryptase that were studied by He and co-workers in work reported in the *Journal*<sup>[19-24]</sup>. Other potent mediators, including various cytokines and leukotrienes, are synthesized *de novo*, with delayed or rapid kinetics depending on whether gene transcription is or is not required, respectively<sup>[19]</sup>. The process of mast cell activation can be mimicked *in vitro* by artificially stimulating aspects of the signaling cascade. He and co-workers accomplished this by using antibodies directed against the IgE molecule itself, which cause allergen-independent IgE receptor cross-linking, or a calcium ionophore, which causes an increase in the levels of cytoplasmic calcium.

In addition to the immunological activation of mast cells, it has been known for many years that various other substances can initiate or potentiate mast cell mediator release<sup>[25]</sup>. These include neuropeptides, highly basic compounds, peptides from bee venom, and adenosine. The work from He *et al.*, conducted with mast cells isolated from human colonic specimens, extends this list to include histamine and proteases, including trypsin and mast cell tryptase itself<sup>[20-24]</sup>. While these and other investigators had shown that proteases can activate some mast cell populations to release histamine, it was important to demonstrate directly that they were active against human intestinal mast cells due to the known existence of substantial functional and biochemical heterogeneity among mast cells isolated from different tissue sites and from different species<sup>[26-29]</sup>. Moreover, the ability of histamine to activate tryptase release from intestinal mast cells had not previously been demonstrated; rather, in other mast cell populations, histamine has been shown to inhibit mediator release, although others have shown that histamine H<sub>1</sub> receptor antagonists can block mediator secretion from basophils, consistent with the findings of He *et al.*<sup>[30,31]</sup>. The work from He and co-workers also implies important autoregulatory mechanisms that almost certainly contribute to the overall level of mediator release from mast cells *in vivo*. Thus, not only are histamine and tryptase released from mast cells, but they also likely stimulate further mediator release once present in the extracellular space. The concentrations of histamine capable of activating mediator release are well within the range that might be expected in the vicinity of activated mast cells<sup>[20]</sup>. Likewise, the biological significance of the inferred effect of tryptase on mediator release is illustrated by the fact that inhibitors of the proteolytic activity of this enzyme significantly reduce mast cell mediator release evoked by IgE cross-linking<sup>[23]</sup>. Overall, these findings suggest that mast cells participate in a self-perpetuating amplification mechanism that would be capable of sustaining mediator release, at least from the granule-associated pool, until released mediators had been cleared from the area by diffusion or metabolism. However, there also appear to be some "brakes" to the system that would preclude wholly uncontrolled release of the panoply of potent mast cell mediators. For histamine in

particular, the effect on mediator secretion was biphasic, with higher concentrations of the amine less effective than lower ones<sup>[20]</sup>. Thus, released histamine would amplify ongoing mediator release from the cell of origin or others in the neighborhood, but only up to a certain point. The biphasic effect of histamine may also explain differences between the findings of He *et al.* and those reported previously by others<sup>[20,30]</sup>.

The authors also have begun to examine the specific receptors and other mechanisms that contribute to protease-activated mediator release from human colonic mast cells. The process is active rather than cytotoxic, and can be reproduced by peptide agonists specific for a member of a novel class of receptors, the proteinase-activated receptors, or PAR's<sup>[32]</sup>. PAR's are G-protein coupled receptors that are activated by proteolytic cleavage, revealing a tethered ligand. The prototypic member of this class is the thrombin receptor, or PAR-1<sup>[32]</sup>. In addition, PAR-2 has been shown to contribute to inflammatory reactions, including in the intestine, and is activated by both trypsin and tryptase, as well as by synthetic peptides that mimic the sequence of the tethered ligand<sup>[32-36]</sup>. He *et al.* developed evidence that mast cells are likely activated by PAR-2 ligation, such as would be stimulated by release of tryptase itself<sup>[21]</sup>. On the other hand, the receptor subtype mediating the effect of histamine on tryptase release from human colonic mast cells is not yet known.

Some minor caveats should be raised about the studies presented. First, the mast cells used for the experiments were studied as an unpurified preparation in which mast cells constituted only about 5% of the total cell number<sup>[20-24]</sup>. Thus, the effects of either histamine and proteases on mediator release could in fact be indirect, and mediated secondarily by another substance released from a contaminating cell type responsive to either agent. However, this scenario does not necessarily detract from the clinical relevance of the responses studied by He and co-workers, because mast cells are not activated in isolation *in vivo*. Likewise, the presence of PAR-2, although not histamine receptors, has been demonstrated directly on human mast cells in a variety of tissues using immunohistochemistry. Second, the authors speculate that mast cells in the gut wall might constantly be exposed to pancreatic trypsin during the normal process of digestion, and that this might evoke mediator release. However, even if a small proportion of luminal trypsin does leak across the small intestinal epithelium in intact form to encounter subepithelial mast cells, and at concentrations comparable to those needed to activate mast cells *in vitro* (which is unproven at the present time), this is unlikely to occur in the colon, and so the studies would need to be repeated using mast cells isolated from the small intestine to understand fully whether the findings have physiological or pathophysiological relevance. Finally, we know little about the persistence of mast cell mediators in the interstitium following their release. While the concentrations of both histamine and proteases capable of activating colonic mast cells are at least theoretically within the biological range immediately following degranulation, it is unknown whether these concentrations remain elevated for long enough to contribute significantly to amplifying subsequent mediator release.

The caveats notwithstanding, the studies of He *et al.* enhance our understanding of the possible roles of mast cells in initiating and/or perpetuating intestinal disorders, including inflammatory bowel diseases and peptic ulcer disease. Knowledge of the mechanisms that regulate mediator release from intestinal mast cells specifically should aid in our ability to modulate the activity of this cell type, with potential therapeutic benefits given the wide range of adverse effects of released mediators. Indeed, He and co-workers themselves suggest that proteinase inhibitors might be attractive targets for drug development, although the biological actions of such

compounds would almost certainly extend beyond simply an effect on mast cell activation, given the wide distribution of PAR's<sup>[23]</sup>. Further, the findings may shed light on pathogenic mechanisms in diseases not currently appreciated as being dependent on mast cells and their mediators. For example, colon cancer cells often release novel trypsins into their environment, and this in turn could conceivably account for the fact that mast cells are often observed at the margins of tumors examined histologically, based on the possibility that such cells are chronically activated by the tumor microenvironment<sup>[37-39]</sup>. Overall, progress in this field should be expected to improve the understanding and treatment of a whole host of digestive disorders.

## REFERENCES

- 1 **Galli SJ.** The Paul Kallos Memorial Lecture. The mast cell: a versatile effector cell for a challenging world. *Int Arch Allergy Immunol* 1997; **113**: 14-22
- 2 **Wershil BK.** Mast cell-deficient mice and intestinal biology. *Am J Physiol Gastrointest Liver Physiol* 2000; **278**: G343-G348
- 3 **Brandt EB, Strait RT, Hershko D, Wang Q, Muntel EE, Scribner TA, Zimmermann N, Finkelman FD, Rothenberg ME.** Mast cells are required for experimental oral allergen-induced diarrhea. *J Clin Invest* 2003; **112**: 1666-1677
- 4 **Barrett KE.** Immune regulation of intestinal ion transport: implications for inflammatory diarrhea. *Progr Inflamm Bowel Dis* 1991; **12**: 8-11
- 5 **Barrett KE, Metcalfe DD.** The mucosal mast cell and its role in gastrointestinal allergic disease. *Clin Rev Allergy* 1984; **2**: 39-53
- 6 **Chadwick VS, Chen W, Shu D, Paulus B, Betwaite P, Tie A, Wilson I.** Activation of the mucosal immune system in irritable bowel syndrome. *Gastroenterology* 2002; **122**: 1778-1783
- 7 **Myers CP, Hogan DL, Yao B, Koss M, Isenberg JJ, Barrett KE.** Inhibition of rabbit duodenal bicarbonate secretion by pro-ulcerogenic agents: histamine-dependent and -independent effects. *Gastroenterology* 1998; **114**: 527-535
- 8 **He SH.** Key role of mast cells and their major secretory products in inflammatory bowel disease. *World J Gastroenterol* 2004; **10**: 309-318
- 9 **Gelbmann CM, Barrett KE.** Role of inflammatory cell types In: Scholmerich J, Kruis W, Goebell H, Hohenberger W, Gross V, eds. *Inflammatory Bowel Diseases - Pathophysiology as Basis of Treatment*. Lancaster, UK: Kluwer Academic 1993: 62-79
- 10 **Gelbmann CM, Barrett KE.** Role of histamine in a rat model of colitis. *Inflamm Res* 1995; **44**: 386-392
- 11 **Perdue MH, Masson S, Wershil BK, Galli SJ.** Role of mast cells in ion transport abnormalities associated with intestinal anaphylaxis. Correction of the diminished secretory response in genetically mast cell-deficient *W/W<sup>v</sup>* mice by bone marrow transplantation. *J Clin Invest* 1991; **87**: 687-693
- 12 **Perdue MH, McKay DM.** Integrative immunophysiology in the intestinal mucosa. *Am J Physiol* 1994; **267**: G151-G165
- 13 **Siddiqui AA, Miner PB Jr.** The role of mast cells in common gastrointestinal diseases. *Curr Allergy Asthma Rep* 2004; **4**: 47-54
- 14 **Mekori YA, Metcalfe DD.** Mast cells in innate immunity. *Immunol Rev* 2000; **173**: 131-140
- 15 **Malaviya R, Abraham SN.** Mast cell modulation of immune responses to bacteria. *Immunol Rev* 2001; **179**: 16-24
- 16 **Bauer O, Razin E.** Mast cell-nerve interactions. *News Physiol Sci* 2000; **15**: 213-218
- 17 **Sada K, Yamamura H.** Protein tyrosine kinases and adaptor proteins in FcεRI-mediated signaling in mast cells. *Curr Mol Med* 2003; **3**: 85-94
- 18 **Barker SA, Lujan D, Wilson BS.** Multiple roles for PI 3-kinase in the regulation of PLCγ activity and Ca<sup>2+</sup> mobilization in antigen-stimulated mast cells. *J Leukocyte Biol* 1999; **65**: 321-329
- 19 **Schwartz LB.** Mast cells: function and contents. *Curr Opin Immunol* 1994; **6**: 91-97
- 20 **He SH, Xie H.** Modulation of tryptase secretion from human colon mast cells by histamine. *World J Gastroenterol* 2004; **10**: 323-326

- 21 **He SH**, He YS, Xie H. Activation of human colon mast cells through proteinase activated receptor-2. *World J Gastroenterol* 2004; **10**: 327-331
- 22 **He SH**, Xie H, He YS. Induction of tryptase and histamine release from human colon mast cells by IgE dependent or independent mechanisms. *World J Gastroenterol* 2004; **10**: 319-322
- 23 **He SH**, Xie H. Inhibition of tryptase release from human colon mast cells by protease inhibitors. *World J Gastroenterol* 2004; **10**: 332-336
- 24 **He SH**, Xie H. Modulation of histamine release from human colon mast cells by protease inhibitors. *World J Gastroenterol* 2004; **10**: 337-341
- 25 **Stassen M**, Hultner L, Schmitt E. Classical and alternative pathways of mast cell activation. *Crit Rev Immunol* 2002; **22**: 115-140
- 26 **He SH**, Walls AF. Human mast cell tryptase: a potent stimulus of microvascular leakage and mast cell activation. *Eur J Pharmacol* 1997; **328**: 89-97
- 27 **He SH**, Gaça MDA, Walls AF. A role for tryptase in the activation of human mast cells: modulation of histamine release by tryptase and inhibitors of tryptase. *J Pharmacol Exp Ther* 1998; **286**: 289-297
- 28 **He SH**, Xie H, He YS. Effect of a proteinase-activated receptor-2 (PAR2) agonist on tryptase release from human mast cells. *Acta Physiol Sin* 2002; **54**: 531-534
- 29 **Barrett KE**, Pearce FL. Heterogeneity of mast cells In: Uvnas B, ed. *Histamine and Histamine Antagonists*. Berlin:Springer Verlag 1991: 93-117
- 30 **Weltman JK**. Histamine as a regulator of allergic and asthmatic inflammation. *Allergy Asthma Proc* 2003; **24**: 227-229
- 31 **McGlashan D Jr**. Histamine: a mediator of inflammation. *J Allergy Clin Immunol* 2003; **112**(4 Suppl): S53-S59
- 32 **Schmidlin F**, Bunnett NW. Protease-activated receptors: how proteases signal to cells. *Curr Opin Pharmacol* 2001; **1**: 575-582
- 33 **Kawabata A**. Gastrointestinal functions of proteinase-activated receptors. *Life Sci* 2003; **74**: 247-254
- 34 **Cenac N**, Garcia-Villar R, Ferrier L, Larauche M, Vergnolle N, Bunnett NW, Coelho AM, Fioramonti J, Bueno L. Proteinase-activated receptor 2-induced colonic inflammation in mice: possible involvement of afferent neurons, nitric oxide, and paracellular permeability. *J Immunol* 2003; **170**: 4296-4300
- 35 **Cottrell GS**, Amadesi S, Schmidlin F, Bunnett N. Protease-activated receptor 2: activation, signalling and function. *Bioch Soc Trans* 2003; **31**: 1191-1197
- 36 **Cenac N**, Coelho AM, Nguyen C, Compton S, Andrade-Gordon P, MacNaughton WK, Wallace JL, Hollenberg MD, Bunnett NW, Garcia-Villar R, Bueno L, Vergnolle N. Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. *Am J Pathol* 2002; **161**: 1903-1915
- 37 **Yamamoto H**, Iku S, Adachi Y, Imsumran A, Taniguchi H, Noshio K, Min Y, Horiuchi S, Yoshida M, Itoh F, Imai K. Association of trypsin expression with tumor progression and matrilysin expression in human colorectal cancer. *J Pathol* 2003; **199**: 176-184
- 38 **Dimitriadou V**, Koutsilieris M. Mast cell-tumor cell interactions: for or against tumour growth and metastasis? *Anticancer Res* 1997; **17**: 1541-1549
- 39 **McKerrow JH**, Bhargava V, Hansell E, Huling S, Kuwahara T, Matley M, Coussens L, Warren R. A functional proteomics screen of proteases in colorectal cancer. *Mol Med* 2000; **6**: 450-460

Edited by Zhang JZ Proofread by Zhu LH

# IL-10 and its related cytokines for treatment of inflammatory bowel disease

Ming-Cai Li, Shao-Heng He

**Ming-Cai Li, Shao-Heng He**, Allergy and Inflammation Research Institute, Shantou University Medical College, Shantou 515041, Guangdong Province, China

**Supported by** the Li Ka Shing Foundation, Hong Kong, China, No. C0200001

**Correspondence to:** Professor Shao-Heng He, Allergy and Inflammation Research Institute, Shantou University Medical College, 22 Xin Ling Road, Shantou 515041, Guangdong Province, China. shoahenghe@hotmail.com

**Telephone:** +86-754-8900405 **Fax:** +86-754-8900192

**Received:** 2003-10-24 **Accepted:** 2003-12-22

## Abstract

Inflammatory bowel diseases (IBDs), including Crohn's disease and ulcerative colitis are chronic inflammatory disorders of gastrointestinal tract. Although the etiology is incompletely understood, initiation and aggravation of the inflammatory process seem to be due to a massive local mucosal immune response. Interleukin-10 (IL-10) is a regulatory cytokine which inhibits both antigen presentation and subsequent pro-inflammatory cytokine release, and it is proposed as a potent anti-inflammatory biological therapy in chronic IBD. Many methods of IL-10 as a treatment for IBD have been published. The new strategies of IL-10 treatment, including recombinant IL-10, the use of genetically modified bacteria, gelatine microsphere containing IL-10, adenoviral vectors encoding IL-10 and combining regulatory T cells are discussed in this review. The advantages and disadvantages of these IL-10 therapies are summarized. Although most results of recombinant IL-10 therapies are disappointing in clinical testing because of lacking efficacy or side effects, therapeutic strategies utilizing gene therapy may enhance mucosal delivery and increase therapeutic response. Novel IL-10-related cytokines, including IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29, are involved in regulation of inflammatory and immune responses. The use of IL-10 and IL-10-related cytokines will provide new insights into cell-based and gene-based treatment against IBD in near future.

Li MC, He SH. IL-10 and its related cytokines for treatment of inflammatory bowel disease. *World J Gastroenterol* 2004; 10 (5): 620-625

<http://www.wjgnet.com/1007-9327/10/620.asp>

## INTRODUCTION

Inflammatory bowel diseases, including Crohn's disease and ulcerative colitis are chronic inflammatory and frequently relapsing diseases of the gut that ultimately lead to destruction of the intestinal tissue. Over recent decades the incidence of IBD has been rising in the world, and IBD will become increasingly common in Asia, as it has been in the last fifty years in Europe and North America<sup>[1]</sup>. The pathogenesis of IBD likely involves multifactorial interactions among genetic factors, immunological factors and environmental triggers<sup>[2]</sup>.

Recent evidence suggests that a pathologic activation of the mucosal immune system in response to antigens is a key factor in the pathogenesis of IBD. In patients with IBD, a loss of immune tolerance occurs to some, but not all, commensal bacterial antigens coupled with alterations in T-cell regulation leads to relapsing chronic inflammation<sup>[3,4]</sup>. There appear to be distinct pathways of inflammation in Crohn's disease and ulcerative colitis. In patients with Crohn's disease, the patterns of cytokines expressed by mucosal lymphocytes generally tend to be consistent with a T-helper-1 (Th1) response, including an early increase in the expression of interferon (IFN), IL-2, and IL-12, followed by a subsequent increase in tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-18<sup>[5]</sup>. In addition, there also appears to be a compensatory increase in IL-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ) levels. In patients with ulcerative colitis, the pattern of cytokine expression differs from that seen in Crohn's disease, with an increased expression of IL-5, IL-6, IL-10, and IL-13<sup>[5]</sup>.

Current therapy of IBD is neither sufficient nor disease-modifying. Long-term treatment with non-specific anti-inflammatory drugs such as aminosalicylates, corticosteroids and immunosuppressants is often accompanied by undesirable and potentially serious side effects<sup>[6]</sup>. Novel biologically-driven therapies are targeted to specific pathophysiological processes, offering the potential for better treatment outcomes. Several new therapeutic strategies are currently being tested in clinical practice, including recombinant anti-inflammatory cytokines (IFN- $\alpha$ , IL-10, IL-11) and inhibitors of cell adhesion molecules (ICAM), pro-inflammatory cytokines (TNF, IL-12) and their specific monoclonal antibodies or their receptors (TNFR, IL-6R)<sup>[2]</sup>.

The pathogenesis of IBD is characterized by an imbalanced activation of Th1- and Th2-lymphocytes. IL-10 represents an anti-inflammatory cytokine which downregulates the production of Th1-derived cytokines. The relative deficiency of IL-10 in patients with ulcerative colitis may contribute to persistent inflammatory changes<sup>[7]</sup>. IL-10-deficient mice (IL-10(-/-)) spontaneously develop intestinal inflammation characterized by discontinuous transmural lesions affecting the small and large intestines and by dysregulated production of proinflammatory cytokines, indicating that endogenous IL-10 is a central regulator of the mucosal immune response<sup>[8]</sup>. IL-10 is in clinical trial as an anti-inflammatory therapy for IBD and various autoimmune diseases such as psoriasis, rheumatoid arthritis, and multiple sclerosis. This review describes the current state of knowledge regarding IL-10-directed biological therapies in IBD.

## BIOLOGICAL CHARACTERISTICS AND FUNCTIONS OF IL-10

IL-10 was first identified in 1989 as a cytokine synthesis inhibitor produced by a subset of murine T lymphocytes termed T-helper-2 (Th2) cells. It is an 18.5 ku cytokine with a broad immunoregulatory activity. It plays the role of a Th2 cell-derived cytokine, and is produced by several cell types, including monocytes, macrophages, T lymphocytes, B cells, dendritic cells, mast cells and various tumour cell lines. Its main biological functions seem to limit and terminate the

inflammatory responses, block the proinflammatory cytokine secretion and regulate the differentiation and proliferation of several immune cells such as T cells, B cells, natural killer cells, antigen-presenting cells, mast cells, and granulocytes<sup>[9]</sup>. IL-10 acts on specific IL-10 receptors that have now been cloned, although the signal transduction pathways and kinases that lead to the widespread anti-inflammatory actions of this cytokine are not yet well understood.

Human IL-10 binds as a 2-fold symmetric homodimer to a functional tetrameric complex of two receptors<sup>[10]</sup>, consisting of two  $\alpha$ - or R1 chains which bind to IL-10, and of two CRF2-4 chains ( $\beta$ - or R2) which initiate the IL-10-induced signal transduction events. CRF2-4 is a member of the class II cytokine receptor family (CRF2), which includes the IFN receptors and is encoded by the CRFB4 gene on chromosome 21<sup>[11]</sup>. IL-10R generates its signals through the JAK1-STAT3 pathway and activates the SOCS-3 gene (suppressor of cytokine signalling-3) of which the expression results in inhibition of JAK/STAT-dependent signalling and the expression of many genes in the cells<sup>[12]</sup>. Some of the actions of IL-10 can be explained by an inhibitory effect on the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B), but this does not account for all effects as IL-10 is very effective on inhibiting IL-5 transcription which is independent of NF- $\kappa$ B<sup>[13]</sup>.

The immunoregulatory activity of IL-10 is based upon its ability to inhibit both cytokine synthesis and antigen presentation. IL-10 inhibits the synthesis of proinflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IL-6), and the Th2 cell-derived cytokines (IL-4 and IL-5)<sup>[13]</sup>. IL-10 also inhibits chemokines such as monocyte inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), RANTES, IL-8 and eotaxin<sup>[14]</sup>, as well as the expression of inflammatory enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) in macrophages, the proliferation of CD4<sup>+</sup> T lymphocytes by inhibiting IL-2 release and reducing expression of major histocompatibility complex (MHC) class II molecules and the costimulatory molecules B7-1, B7-2, and low-affinity IgE receptors (CD23) in antigen-presenting cells, thus effectively blocking allergen presentation by mononuclear cells and dendritic cells to T cells. In addition, IL-10 also increases the expression of several anti-inflammatory proteins, including IL-1 receptor antagonist, soluble TNF- $\alpha$  receptor and tissue inhibitor of matrix metalloproteinases. Although many of the inhibitory effects of IL-10 on T lymphocytes are secondary to this reduction in monocyte-derived pro-inflammatory cytokines, it also has a direct regulatory effect on T-cell differentiation and proliferation by inhibiting IL-2 and IFN- $\gamma$  release from activated T-cell clones<sup>[15]</sup>.

However, the biology of IL-10 is highly complex. In addition to the down-regulation of immunity, both human IL-10 and murine IL-10 exert immunostimulatory effects by up-regulating MHC class II expression on B lymphocytes, and inducing cytotoxic T-cell differentiation. To complicate the issue, there is strong homology in the complementary DNA of human and murine IL-10 with an open reading frame sequence in the Epstein-Barr virus (human herpes virus type 4) genome, termed viral IL-10. Whereas viral IL-10 shares many of the immunoregulatory properties of human IL-10, it lacks immunostimulatory effects.

## IL-10 THERAPY FOR IBD

### Recombinant IL-10

The therapeutic experience of IL-10 in animal models of colitis is very encouraging. *In vitro* studies have shown that exogenous IL-10 can down-regulate the enhanced pro-inflammatory cytokine release from lamina propria mononuclear cells isolated from patients with Crohn's disease. Early clinical studies in untreated, as well as steroid-refractory Crohn's disease

patients, suggested that IL-10 might have strong potential in the treatment of human diseases. Colombel *et al*<sup>[16]</sup> performed a double blind controlled trial to evaluate the safety and tolerance of recombinant human interleukin 10 (IL-10, Tenovil) in subjects operated on for Crohn's disease. Tenovil treatment for 12 consecutive weeks in patients with Crohn's disease after intestinal resection was safe and well tolerated. No evidence of prevention of endoscopic recurrence of Crohn's disease by Tenovil was observed.

Unfortunately, this enthusiasm was dampened by the results of 2 multinational multicenter studies reporting the efficacy and safety of daily subcutaneous injections of recombinant human IL-10(rhuIL-10) in patients with moderately active and steroid-refractory Crohn's disease<sup>[17,18]</sup>. Fedorak *et al*<sup>[17]</sup> reported a 24-week double-blind placebo controlled study of 95 patients with moderately active Crohn's disease randomized to receive subcutaneous rhuIL-10 at 1 of 4 doses (rhuIL-10:1, 5, 10, or 20  $\mu$ g/kg) or placebo daily. After a 28-day treatment period, a modest response was seen in the group receiving the 5  $\mu$ g/kg dose, whereby 23.5% of patients ( $n=17$ ) showed improvement compared with 0% of patients treated with placebo ( $n=23$ ). Interestingly, higher doses were less effective, although there was no difference in reported adverse events between doses. During the course of a 20-week follow-up period, disease recurrence requiring therapeutic intervention occurred in 70% of placebo-treated patients compared to 55% of the rhuIL-10-treated group<sup>[17]</sup>.

In the study by Schreiber *et al*<sup>[18]</sup> involving 329 therapy refractory patients with active Crohn's disease, no significant differences in the induction of clinical remission were seen between placebo and rhuIL-10 at any dose (1, 4, 8, and 20  $\mu$ g/kg), although clinical improvement was achieved in 46% of patients in the 8  $\mu$ g/kg group compared with 27% of patients receiving placebo ( $P=0.03$ ). The authors also determined the nuclear levels of transcription factor NF- $\kappa$ B p65 and cytoplasmic concentrations of its inhibitor I $\kappa$ B $\alpha$  in ileocolonic biopsy specimens. They noticed significant differences between patients who responded to rhuIL-10 treatment and those who did not, implicating the inhibition of NF- $\kappa$ B nuclear translocation as one possible mechanism of the action of this therapy<sup>[18]</sup>.

Recent studies linking high systemic concentrations of IL-10 with headache, fever<sup>[19]</sup>, and anemia<sup>[20]</sup> have further aroused the enthusiasm for IL-10 as a therapeutic drug in treatment of IBD. Indeed, the biology of IL-10 is complex. In addition to its effects in down-regulating pro-inflammatory events, IL-10 has been shown to exert immunostimulatory effects by up-regulating MHC II expression on B lymphocytes and inducing cytotoxic T-cell differentiation. Clinical data support these immunostimulatory effects of IL-10 at high systemic doses. Tilg *et al*<sup>[19]</sup> reported that patients with Crohn's disease receiving rhIL-10 at high doses experienced a significant increase in serum neopterin and phytohaemagglutinin (PHA) induced IFN- $\gamma$  production from whole blood cells. This phenomenon may be responsible for the lack of efficacy of high doses of IL-10 in the treatment of Crohn's disease<sup>[19]</sup>. In fact, the emerging picture regarding the *in vivo* effects of IL-10 is one of both the anti- and pro-inflammatory effects, depending on the local concentrations of IL-10 achieved, the types of antigens present in the microenvironment, and the activation state of the immune cells in the vicinity. It is possible that high systemic doses of IL-10 alter the balance between its immunoregulatory and immunostimulatory effects, which would explain the bell-shaped curve in therapeutic efficacy reported in the clinical trials. In addition, it is entirely possible that the pharmacodynamics of daily systemic IL-10 administration does not allow for efficient delivery of the cytokine to the local sites of inflammation. The serum half-life of IL-10 is between 1.1 and 2.6 hours, thus, the cytokine may be cleared before

reaching its target.

### **Treatment by *Lactococcus lactis* secreting IL-10**

IBD in humans responds to high dose of intravenous interleukin 10, but this cannot be administered orally because stomach acid destroys it. Recently, studies have been published that looked at alternative methods of delivery of IL-10. The first study used the genetically engineered bacterium, *Lactococcus lactis* (*L. lactis*), as a delivery vehicle for IL-10. Steidler *et al*<sup>[21]</sup> hypothesised that an oral delivery system that effectively bypassed the stomach might provide a treatment which would cut the dose of IL-10 required and also reduce the risk of systemic side effects. Steidler and his colleagues<sup>[21]</sup> have engineered a non-pathogenic bacterium that produces interleukin 10, an anti-inflammatory cytokine that reduces inflammatory colitis. They then established two different mice models of inflammatory bowel disease. In the first model chronic colitis was induced by feeding dextran sulphate sodium to the mice over several weeks. When the recombinant bacteria were introduced into the mice stomachs, they survived the acid environment and released interleukin 10 in the colon. The second mouse model had its interleukin 10 gene knocked out. The resultant mice were shown to develop IBD spontaneously within 8 weeks of life. When the team administered the genetically engineered *L. lactis* to these mice at 3 weeks of age, colitis did not occur in the first place. They showed that daily intragastric administration of IL-10-secreting *L. lactis* caused a 50% reduction in colitis in mice treated with dextran sulfate sodium and prevented the onset of colitis in IL-10(-/-) mice. High serum concentrations of interleukin 10 were not found, despite good uptake by inflamed cells of the gut mucosa. This approach may lead to better methods for cost-effective and long-term management of IBD in humans. Because the genetically engineered bacterium survives stomach acid, the cytokine can be delivered directly to the inflammatory target in the colon. This could reduce the dose needed and any systemic effects.

However, the release of such genetically modified organisms through clinical use raises safety concerns. Steidler *et al*<sup>[22]</sup> replaced the thymidylate synthase gene *thyA* of *L. lactis* with a synthetic human IL10 gene to address this problem. This *thyA*-hIL10<sup>+</sup> *L. lactis* strain produced human IL-10(hIL-10), and when deprived of thymidine or thymine, its viability dropped by several orders of magnitude, essentially preventing its accumulation in the environment. The biological containment system and the bacterium's capacity of secreting hIL-10 were validated *in vivo* in pigs. This approach is a promising one for transgene containment because, in the unlikely event that the engineered *L. lactis* strains acquired an intact *thyA* gene from a donor such as *L. lactis* subsp. *cremoris*, the transgene would be eliminated from the genome.

Two technical hitches must be ironed out, however, before human trials can be considered. Human interleukin 10 is slightly different from murine interleukin 10, so the *L. lactis* has to be re-engineered. The second obstacle is that human bile is stronger than mouse bile and is likely to kill the bacteria as they pass through the stomach and duodenum<sup>[23]</sup>.

### **Gelatin microspheres containing interleukin-10**

IL-10 is an anti-inflammatory cytokine that suppresses the T helper 1 immune response and down-regulates macrophages and monocytes. The therapeutic effect of systemic administration of IL-10 for patients with IBD, however, has not been satisfactory. Several studies have indicated that active monocytes, such as macrophages and T cells, play an important role in the pathogenesis of chronic human IBD, although the etiology remains unclear. Manipulation of these cells appears essential for the treatment of patients with IBD. Recently,

considerable attention has been paid to the use of polymer microspheres for the sustained release of various drugs and the targeting of therapeutic agents to their sites of action.

Nakase *et al*<sup>[24,25]</sup> developed gelatin microspheres (GM) containing IL-10 (GM-IL-10), which can be released sustainably to a local site without losing bioactivity. They administered these microspheres to IL-10 knocked out mice rectally to investigate whether this treatment could ameliorate colitis. Colonic inflammation in mice treated with GM-IL-10 was remarkably reduced compared to those treated with IL-10 alone. Macroscopic and microscopic examination revealed marked improvement of colitis in IL-10(-/-) mice treated with GM-IL-10. mRNA expression of IL-12 in Mac-1-positive cells in GM-IL-10-treated mice was significantly decreased compared with that in the mice treated with IL-10 alone. CD40 expression in Mac-1-positive cells in GM-IL-10-treated mice was decreased more prominently than that in mice treated with IL-10 alone. The therapeutic effects of GM-IL-10 were associated with decreased expression of IL-12 mRNA and down-regulation of CD40 expression in Mac-1-positive cells. Additionally, intestinal administration of these microspheres significantly improved colitis with a decrease in histological score, myeloperoxidase activity, and nitric oxide production compared with those treated with free agents. Gene expressions of TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$  were down-regulated in treated animals. Serum IL-10 levels and systemic macrophages were unchanged after treatment<sup>[26]</sup>.

These data suggest that local macrophages in the intestine play a critical role in the initiation of chronic colitis in the animal model of IBD. A drug delivery system using these microspheres containing immunomodulatory IL-10 (GM-IL-10) might be useful for treatment of patients with IBD.

### **Gene therapy**

IL-10 is an endogenous anti-inflammatory and immunomodulatory cytokine that has been shown to prevent inflammation and injury in several animal studies, however clinical IL-10 treatment remains insufficient because of difficulties in the route of IL-10 administration and its biological half-life. It may be possible to use replication-deficient adenoviral vectors to deliver the IL-10 gene directly to gastrointestinal epithelial cells. This would lead to localized high level IL-10 release for a short duration that is determined by the lifetime of the infected cells. IL-10 adenoviral gene therapy has proved very successful in murine models of rheumatoid arthritis<sup>[27]</sup>, a condition with many immunological similarities to Crohn's disease. Previous studies have demonstrated that adenoviral vectors, when delivered by rectal infusion, could infect intestinal epithelial cells<sup>[28]</sup>. This approach may be limited by the host anti-adenoviral immune response, that has limited gene expression and prevented re-treatment with other adenoviral vectors<sup>[29]</sup>. However, there is evidence that the delivery of immunoregulatory genes, such as IL-10, would diminish both the cell-mediated and humoral anti-adenoviral responses<sup>[30,31]</sup>.

Barbara *et al*<sup>[32]</sup> reported that gene transfer was achieved by intraperitoneal injection of non-replicating human type 5 adenovirus bearing IL-10 gene, either 24 hours before or one hour after intrarectal administration of dinitrobenzene sulphonic acid in rats. Colonic damage and inflammation were assessed macroscopically and by measuring the myeloperoxidase activity and leukotriene B4 concentrations. Gene transfer increased IL-10 protein in serum for up to six days. IL-10 gene transfer prior to colitis improved colitis macroscopically and histologically, and significantly reduced colonic myeloperoxidase activity and leukotriene B4 concentrations. In contrast, IL-10 gene transfer after the onset of colitis had no beneficial effect. Lindsay *et al*<sup>[33]</sup> showed that local adenoviral vectors encoding IL-10 (AdvmuIL-10) reverse

colitis in IL-10<sup>-/-</sup> mice without systemic effects seen after intravenous administration.

Enhanced expression of MAdCAM-1 (mucosal addressin cell adhesion molecule-1) is associated with the onset and progression of IBD. Sasaki *et al.*<sup>[34]</sup> reported transfection of the IL-10 vector into endothelial cell cultures significantly reduced TNF- $\alpha$  induction, MAdCAM-1 dependent lymphocyte adhesion (compared to non-transfected cells). IL-10 transfected endothelial cells expressed less than half (46 $\pm$ 6.6%) of the MAdCAM-1 induced by TNF- $\alpha$  (set as 100%) in non-transfected (control) cells.

These results suggest that gene therapy strategies using plasmid IL-10 vectors or an adenovirus-IL-10 construct may prove to be a potent approach to the treatment of chronic inflammatory diseases such as Crohn's disease.

### Combining regulatory T cells and IL-10

T cells, and in particular, regulatory T (Treg) cells, play a pivotal role in the control of intestinal inflammation<sup>[35,36]</sup>. Regulatory cells lacking anti-inflammatory cytokine IL-10 were unable to inhibit IBD, showing that IL-10 was required for the protective effects of lymphocytes in this setting<sup>[37]</sup>.

van Montfrans *et al.*<sup>[38,39]</sup> presented a novel method of IL-10 delivery to intestinal mucosal tissue by the use of transduced T cells. In these studies, the authors demonstrated that T lymphocytes could be engineered by retrovirus construct transduction to express high levels of IL-10 upon activation. With the goal of providing long-term therapy for Crohn's disease, peripheral blood mononuclear cells were obtained from healthy adults and transduced with a retroviral vector containing IL-10 and green fluorescent protein (GFP). These CD4<sup>+</sup> T lymphocytes responded to CD3/CD28 stimulation with a 6-fold increase in IL-10 production that was shown to be biologically active. Transduced cells had high expressions of the mucosal integrin  $\alpha 4\beta 7$ , and displayed efficient binding to MAdCAM-1 expressing cells *in vivo*, suggesting that they would home to gut mucosa. Importantly, albeit *in vitro*, cells remained stably transfected for up to 4 months. In the classic CD45RB<sup>high</sup> model, these IL-10 transduced CD4<sup>+</sup> cells were able to effectively prevent the development of colitis, even when given up 14 days after the transfer of the CD45RB<sup>high</sup> cells. Although circulating levels of IL-10 were not detectable, IL-10-GFP encoding messenger RNA was detected in colons of mice 15 weeks after cell transfer, and IL-10 was detected in the intestinal draining lymph nodes, spleen, and colon, indicating that the IL-10-GFP transduced CD4<sup>+</sup> cells persisted *in vivo* and migrated into the large intestine. The mice receiving IL-10 transduced CD4<sup>+</sup> cells exhibited a decrease in the production of TNF- $\alpha$  in the colon and a decreased production of IFN- $\gamma$  and/or TNF- $\alpha$  in the intestinal draining lymph nodes. These results indicate that the transferred CD4<sup>+</sup> cells are stimulated *in vivo* and therapeutically effective.

One of the advantages of using T cells as delivery vehicles is the likelihood that the cytokine of interest, in this case, IL-10, would primarily be released only on activation in local sites of inflammation, which would avoid the side effects known to be associated with high systemic levels and may further induce the development of immune-suppressor Treg cells in the area of inflammation. Secondly, the use of *ex vivo* transduction methodology would prevent systemic exposure to the retroviral vector, but still ensure sustained gene expression. However, the question remains, will even local delivery of IL-10 be effective in patients with IBD? IL-10 certainly exhibits potent inhibitory effect on the activation and effector functions of T cells, monocytes, and dendritic cells, all of which are key players in intestinal inflammation. However, the colon from a patient with Crohn's disease already secreted higher levels of IL-10 compared with control colons<sup>[40]</sup> and mononuclear cells

isolated from the ileum of patients with Crohn's disease appeared to be nonresponsive to IL-10<sup>[41]</sup>. These findings suggest that even if IL-10 is delivered directly to the intestinal mucosa, it may not be therapeutic in Crohn's disease. This is mirrored by effects both in the IL-10 gene-deficient mouse chronic colitis model and in the dinitrobenzene sulphonic acute model of colitis<sup>[42]</sup> where IL-10 is able to prevent the development of colitis if given before inflammation develops, but is ineffective if given either exogenously or via gene transfer, once inflammation has become established. However, although IL-10 therapy may be ineffectual in treating active diseases, there may still exist a role for such therapy as maintenance treatment in Crohn's disease. Low ileal IL-10 concentrations have been shown to predict relapse after ileocecal resection<sup>[43]</sup>. In addition, it may be that only certain subgroups of Crohn's disease patients respond to IL-10 therapy, whether it is delivered systemically or locally. It is clear that even among patients with similar clinical presentations, there exists a large diversity of patterns of immune responses to environmental and bacterial antigens<sup>[44]</sup>. It is not known how patients with these types of immune reactivities respond to different therapies.

### CONCLUSION AND PERSPECTIVES

IL-10 is a pivotal cytokine in the control of intestinal inflammation. Several clinical trials<sup>[45]</sup> have demonstrated that daily systemic rhuIL-10 injections were safe and well tolerated with modest efficacy in Crohn's disease, but clinical trials with rhuIL-10 in Crohn's disease were disappointing, although some patients showed healing of intestinal mucosa. With the administered dose of IL-10 in the clinical trials, the ultimate local IL-10 concentrations in the intestine could be too low to result in downregulation of inflammation. Furthermore, higher doses of systemically administered IL-10 (which were also used in the clinical trials) might be detrimental rather than helpful<sup>[46]</sup>. But it is possible that response to IL-10 is limited to a subgroup of patients, and perhaps delivering the IL-10 in a more sustained and focused manner would prove to be effective. These may be the several reasons why this is not the end of the road for rhuIL-10 therapy in Crohn's disease<sup>[29]</sup>. Recently, some novel alternative approaches, including the use of genetically modified *Lactococcus lactis*, gelatine microsphere containing IL-10, adenoviral vectors encoding IL-10 and combining regulatory T cells, may ensure that delivery of IL-10 is indeed local, tissue-specific, and therapeutic. These therapeutic approaches caused a significant reduction in intestinal inflammation in different mouse models, and they might be useful for treatment of human IBD. However, there is still a long way to go until these approaches can be evaluated in clinical studies.

New mammalian genes that encode IL-10-related cytokines have been described as the result of experiments using cDNA subtraction cloning focusing on melanocyte differentiation (*IL24*)<sup>[47]</sup>, virus-induced T-cell transformation (*IL26*)<sup>[48]</sup> or IL-9-mediated gene induction (*IL22*)<sup>[49]</sup>. The sequencing and annotation of the human genome could lead to the identification of five additional IFN- or IL-10-related genes<sup>[50-53]</sup>: *IL19*, *IL20*, *IL28A* (also known as *IFN12*), *IL28B* (also known as *IFN13*) and *IL29* (also known as *IFN11*). Similar to IL-10, these IL-10-related cytokines are  $\alpha$ -helical proteins with similar cysteine localizations, whose amino acid sequences are about 20-30% identical. IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B and IL-29 are grouped together on the basis of structural homologies, indicating that these genes are derived from common ancestors<sup>[54]</sup>. All these new IL-10 supfamily member cytokines are strongly involved in immune regulation and inflammatory response, further studies will provide a better understanding of potential therapeutic utilities, some of them



may reduce adverse side effects and/or increase the efficacy typically seen in IL-10 therapy for IBD in the future.

## REFERENCES

- Rampton DS, Phil D. New treatments for inflammatory bowel disease. *World J Gastroenterol* 1998; **4**: 369-376
- Sandborn WJ, Targan SR. Biologic therapy of inflammatory bowel disease. *Gastroenterology* 2002; **122**: 1592-1608
- Strober W, Fuss IJ, Blumberg RS. Immunology of mucosal models of inflammation. *Ann Rev Immunol* 2002; **20**: 495-549
- Landers CJ, Cohavy O, Misra R, Yang H, Lin Y, Braun J, Targan SR. Selected loss of tolerance evidenced by Crohn's disease-associated immune responses to auto and microbial antigens. *Gastroenterology* 2002; **123**: 689-699
- Madsen K. Combining T cells and IL-10: A new therapy for Crohn's disease? *Gastroenterology* 2002; **123**: 2140-2144
- Das KM, Farag SA. Current medical therapy of inflammatory bowel disease. *World J Gastroenterol* 2000; **6**: 483-489
- Ishizuka K, Sugimura K, Homma T, Matsuzawa J, Mochizuki T, Kobayashi M, Suzuki K, Otsuka K, Tashiro K, Yamaguchi O, Asakura H. Influence of interleukin-10 on the interleukin-1 receptor antagonist/interleukin-1 beta ratio in the colonic mucosa of ulcerative colitis. *Digestion* 2001; **63**: 22-27
- Rennick DM, Fort MM. Lessons from genetically engineered animal models. XII. IL-10-deficient (IL-10<sup>-/-</sup>) mice and intestinal inflammation. *Am J Physiol Gastrointest Liver Physiol* 2000; **278**: G829-833
- Asadullah K, Sterry W, Volk HD. Interleukin-10 Therapy—Review of a New Approach. *Pharmacol Rev* 2003; **55**: 241-269
- Donnelly RP, Dickensheets H, Finbloom DS. The interleukin-10 signal transduction pathway and regulation of gene expression in mononuclear phagocytes. *J Interferon Cytokine Res* 1999; **19**: 563-573
- Reboul J, Gardiner K, Monneron D, Uze G, Lutfalla G. Comparative genomic analysis of the interferon/interleukin-10 receptor gene cluster. *Genome Res* 1999; **9**: 242-250
- Riley JK, Takeda K, Akira S, Schreiber RD. Interleukin-10 receptor signaling through the JAK-STAT pathway. Requirement for two distinct receptor-derived signals for anti-inflammatory action. *J Biol Chem* 1999; **274**: 16513-16521
- Staples KJ, Bergmann M, Barnes PJ, Newton R. Stimulus-specific inhibition of IL-5 by cAMP-elevating agents and IL-10 reveals differential mechanisms of action. *Biochem Biophys Res Commun* 2000; **273**: 811-815
- Chung KF, Patel HJ, Fadlon EJ, Rousell J, Haddad EB, Jose PJ, Mitchell J, Belvisi M. Induction of eotaxin expression and release from human airway smooth muscle cells by IL-1 $\beta$  and TNF- $\alpha$ : effects of IL-10 and corticosteroids. *Br J Pharmacol* 1999; **127**: 1145-1150
- Ebert EC. IL-10 enhances IL-2-induced proliferation and cytotoxicity by human intestinal lymphocytes. *Clin Exp Immunol* 2000; **119**: 426-432
- Colombel JF, Rutgeerts P, Malchow H, Jacyna M, Nielsen OH, Rask-Madsen J, Van Deventer S, Ferguson A, Desreumaux P, Forbes A, Geboes K, Melani L, Cohard M. Interleukin 10 (Tenovil) in the prevention of postoperative recurrence of Crohn's disease. *Gut* 2001; **49**: 42-46
- Fedorak RN, Gangl A, Elson CO, Rutgeerts P, Schreiber S, Wild G, Hanaver SB, Kilian A, Cohard M, Le Beut A, Feagan B. Recombinant human interleukin 10 in the treatment of patients with mild to moderately active Crohn's disease. *Gastroenterology* 2000; **119**: 1473-1482
- Schreiber S, Fedorak RN, Nielsen OG, Wild G, Williams CN, Nikolaus S, Jacyna M, Lashner BA, Gangl A, Rutgeerts P, Isaacs K, van Deventer SJ, Koningsberger JC, Cohard M, Le Beut A, Hanaver SB. Safety and efficacy of recombinant human interleukin 10 in chronic active Crohn's disease. *Gastroenterology* 2000; **119**: 1461-1472
- Tilg H, van Montfrans C, van den Ende A, Kaser A, van Deventer SJ, Schreiber S, Gregor M, Ludwiczek O, Rutgeerts P, Gasche C, Koningsberger JC, Abreu L, Kuhn I, Cohard M, Le Beut A, Grnt P, Weiss G. Treatment of Crohn's disease with recombinant human interleukin 10 induces the proinflammatory cytokine interferon gamma. *Gut* 2002; **50**: 191-195
- Tilg H, Ulmer H, Kaser A, Weiss G. Role of IL-10 for induction of anemia during inflammation. *J Immunol* 2002; **169**: 2204-2209
- Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* 2000; **289**: 1352-1355
- Steidler L, Neirynck S, Huyghebaert N, Snoeck V, Vermeire A, Goddeeris B, Cox E, Remon JP, Remaut E. Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. *Nat Biotechnol* 2003; **21**: 785-789
- Michie C. Germ therapy with IL-10 to treat inflammatory bowel diseases. *Mol Med Today* 2000; **6**: 416
- Nakase H, Okazaki K, Tabata Y, Ozeki M, Watanabe N, Ohana M, Uose S, Uchida K, Nishi T, Mastuura M, Tamaki H, Itoh T, Kawanami C, Chiba T. New cytokine delivery system using gelatin microspheres containing interleukin-10 for experimental inflammatory bowel disease. *J Pharmacol Exp Ther* 2002; **301**: 59-65
- Nakase H, Okazaki K, Tabata Y, Chiba T. Biodegradable microspheres targeting mucosal immune-regulating cells: new approach for treatment of inflammatory bowel disease. *J Gastroenterol* 2003; **38**: 59-62
- Okazaki K, Nakase H, Watanabe N, Tabata Y, Ikada Y, Chiba T. Intestinal drug delivery systems with biodegradable microspheres targeting mucosal immune-regulating cells for chronic inflammatory colitis. *J Gastroenterol* 2002; **37**: 44-52
- Whalen JD, Lechman EL, Carlos CA, Weiss K, Kovacs I, Glorioso JC, Robbins PD, Evans CH. Adenoviral transfer of the viral IL-10 gene periarticularly to mouse paws suppresses development of collagen-induced arthritis in both injected and uninjected paws. *J Immunol* 1999; **162**: 3625-3632
- Wirtz S, Galle PR, Neurath MF. Efficient gene delivery to the inflamed colon by local administration of recombinant adenoviruses with normal or modified fibre structure. *Gut* 1999; **44**: 800-807
- Lindsay JO, Hodgson H. The immunoregulatory cytokine interleukin-10—a therapy for Crohn's disease? *Aliment Pharmacol Ther* 2001; **15**: 1709-1716
- Minter RM, Rectenwald JE, Fukuzuka K, Tannahill CL, La Face D, Tsai V, Ahmed I, Hutchins E, Moyer R, Copeland EM 3rd, Moldawer LL. TNF-alpha receptor signaling and IL-10 gene therapy regulate the innate and humoral immune responses to recombinant adenovirus in the lung. *J Immunol* 2000; **164**: 443-451
- Chirmule N, Truneh A, Haecker SE, Tazelaar J, Gao G, Raper SE, Hughes JV, Wilson JM. Repeated administration of adenoviral vectors in lungs of human CD4 transgenic mice treated with a nondepleting CD4 antibody. *J Immunol* 1999; **163**: 448-455
- Barbara G, Xing Z, Hogaboam CM, Gaudie J, Collins SM. Interleukin 10 gene transfer prevents experimental colitis in rats. *Gut* 2000; **46**: 344-349
- Lindsay JO, Ciesielski CJ, Scheinin T, Brennan FM, Hodgson HJ. Local delivery of adenoviral vectors encoding murine interleukin 10 induces colonic interleukin 10 production and is therapeutic for murine colitis. *Gut* 2003; **52**: 363-369
- Sasaki M, Jordan P, Houghton J, Meng X, Itoh M, Joh T, Alexander JS. Transfection of IL-10 expression vectors into endothelial cultures attenuates alpha4beta7-dependent lymphocyte adhesion mediated by MAdCAM-1. *BMC Gastroenterol* 2003; **3**: 3
- Singh B, Read S, Asseman C, Malmstrom V, Mottet C, Stephens LA, Stepankova R, Tlaskalova H, Powrie F. Control of intestinal inflammation by regulatory T cells. *Immunol Rev* 2001; **182**: 190-200
- Roncarolo MG, Bacchetta R, Bordinon C, Narula S, Levings MK. Type 1 T regulatory cells. *Immunol Rev* 2001; **182**: 68-79
- Groux H, Powrie F. Regulatory T cells and inflammatory bowel disease. *Immunol Today* 1999; **20**: 442-445
- Van Montfrans C, Hooijberg E, Rodriguez Pena M, de Jong E, Spits H, te Velde A, van Deventer SJH. Generation of regulatory gut-homing human T lymphocytes using *ex vivo* interleukin 10 gene transfer. *Gastroenterology* 2002; **123**: 1877-1888
- Van Montfrans C, Rodriguez Pena M, Pronk I, ten Kate FJW, te Velde AA, van Deventer SJH. Prevention of colitis by interleukin-10 transduced T lymphocytes in the transfer model. *Gastroenterology* 2002; **123**: 1865-1876
- Lindsay JO, Feldmann M, Hodgson H, Brennan F. The immunoregulatory role of interleukin-10 in Crohn's disease (CD)



- cell cultures. *Gastroenterology* 2000; **118**: A110
- 41 **Colpaert S**, Vanstraelen K, Liu Z, Penninckx F, Geboes K, Rutgeerts P, Ceuppens J. Decreased lamina propria effector cell responsiveness to interleukin-10 in ileal Crohn's disease. *Clin Immunol* 2002; **102**: 68-76
  - 42 **Barbara G**, Xing Z, Hogaboam CM, Gauldie J, Collins SM. Interleukin 10 gene transfer prevents experimental colitis in rats. *Gut* 2000; **46**: 344-349
  - 43 **Meresse B**, Rutgeerts P, Malchow H, Dubucquoi S, Dessaint JP, Cohard M, Colombel JF, Desreumaux P. Low ileal interleukin 10 concentrations are predictive of endoscopic recurrence in patients with Crohn's disease. *Gut* 2002; **50**: 25-28
  - 44 **Landers CJ**, Cohavy O, Misra R, Yang H, Lin Y, Braun J, Targan SR. Selected loss of tolerance evidenced by Crohn's disease-associated immune responses to auto and microbial antigens. *Gastroenterology* 2002; **123**: 689-699
  - 45 **Bickston SJ**, Cominelli F. Recombinant interleukin 10 for the treatment of active Crohn's disease: lessons in biologic therapy. *Gastroenterology* 2000; **119**: 1781-1783
  - 46 **Herfarth H**, Scholmerich J. IL-10 therapy in Crohn's disease: at the crossroads. Treatment of Crohn's disease with the anti-inflammatory cytokine interleukin 10. *Gut* 2002; **50**: 146-147
  - 47 **Jiang H**, Lin JJ, Su ZZ, Goldstein NI, Fisher PB. Subtraction hybridization identifies a novel melanoma differentiation associated gene, mda-7, modulated during human melanoma differentiation, growth and progression. *Oncogene* 1995; **11**: 2477-2486
  - 48 **Knappe A**, Hor S, Wittmann S, Fickenscher H. Induction of a novel cellular homolog of interleukin-10, AK155, by transformation of T lymphocytes with herpesvirus saimiri. *J Virol* 2000; **74**: 3881-3887
  - 49 **Dumoutier L**, Louahed J, Renauld JC. Cloning and characterization of IL-10-related T cell-derived inducible factor (IL-TIF), a novel cytokine structurally related to IL-10 and inducible by IL-9. *J Immunol* 2000; **164**: 1814-1819
  - 50 **Gallagher G**, Dickensheets H, Eskdale J, Izotova LS, Mirochnitchenko OV, Peat JD, Vazquez N, Pestka S, Donnelly RP, Kotenko SV. Cloning, expression and initial characterization of interleukin-19 (IL-19), a novel homologue of human interleukin-10 (IL-10). *Genes Immun* 2000; **1**: 442-450
  - 51 **Blumberg H**, Conklin D, Xu WF, Grossmann A, Brender T, Carollo S, Eagan M, Foster D, Haldeman BA, Hammond A, Haugen H, Jelinek L, Kelly JD, Madden K, Maurer MF, Parrish-Novak J, Prunkard D, Sexson S, Sprecher C, Waggie K, West J, Whitmore TE, Yao L, Kuechle MK, Dale BA, Chandrasekhar YA. Interleukin 20: discovery, receptor identification, and role in epidermal function. *Cell* 2001; **104**: 9-19
  - 52 **Sheppard P**, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, Kuestner R, Garrigues U, Birks C, Roraback J, Ostrand C, Dong D, Shin J, Presnell S, Fox B, Haldeman B, Cooper E, Taft D, Gilbert T, Grant FJ, Tackett M, Krivan W, McKnight G, Clegg C, Foster D, Klucher KM. IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nature Immunol* 2003; **4**: 63-68
  - 53 **Kotenko SV**, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H, Donnelly RP. IFN- $\lambda$ s mediate antiviral protection through a distinct class II cytokine receptor complex. *Nature Immunol* 2003; **4**: 69-77
  - 54 **Renauld JC**. Class II cytokine receptors and their ligands: key antiviral and inflammatory modulators. *Nat Rev Immunol* 2003; **3**: 667-676

Edited by Wang XL Proofread by Zhu LH

• ESOPHAGEAL CANCER •

# Long-term effect on carcinoma of esophagus of distal subtotal gastrectomy

Yu-Ping Chen, Jie-Sheng Yang, Di-Tian Liu, Yu-Quan Chen, Wei-Ping Yang

**Yu-Ping Chen, Jie-Sheng Yang, Di-Tian Liu, Yu-Quan Chen, Wei-Ping Yang**, Department of Thoracic Surgery, Tumor Hospital of Shantou University Medical College, Shantou 515031, Guangdong Province, China

**Correspondence to:** Dr. Yu-Ping Chen, Department of Thoracic Surgery, Tumor Hospital of Shantou University Medical College, Shantou 515031, Guangdong Province, China. chenyp@pub.shantou.gd.cn

**Telephone:** +86-754-8630899 **Fax:** +86-754-8630899

**Received:** 2003-05-13 **Accepted:** 2003-06-02

## Abstract

**AIM:** To investigate the surgical treatment and long-term survival for patients with carcinoma of esophagus after distal subtotal gastrectomy.

**METHODS:** Resections of the tumor through left thoracotomy were performed in 85 patients with esophageal carcinoma following distal subtotal gastrectomy. The procedure involved preserving the left short gastric artery and transporting the residual stomach, the spleen and tail of the pancreas into the left thoracic cavity, and using the residual stomach to reconstruct the alimentary tract.

**RESULTS:** The resectable rate was 91.8%, complication rate 10.3%, and no death occurred in the postoperative period. The 1-, 3-, 5-, and 10-year survival rates were 85.7%, 50.7%, 30.6% and 18.8%, respectively.

**CONCLUSION:** Surgical resection is the optimal management method for the patients with esophageal carcinoma after distal subtotal gastrectomy. The reconstruction of digestive tract using anastomosis of the esophagus and the residual stomach is not only simple but also can achieve a better curative effect, promoting the digestive function and improving the quality of life.

Chen YP, Yang JS, Liu DT, Chen YQ, Yang WP. Long-term effect on carcinoma of esophagus of distal subtotal gastrectomy. *World J Gastroenterol* 2004; 10(5): 626-629 <http://www.wjgnet.com/1007-9327/10/626.asp>

## INTRODUCTION

The primary management of esophageal carcinoma is surgical resection, and the stomach is the organ most often chosen for substitution following removal of esophagus for carcinoma. However, following the resection of esophageal carcinoma after distal subtotal gastrectomy, the replacement of esophagus mostly selected other organs, such as jejunum or colon<sup>[1-11]</sup>. Since we first applied this new technique to use the residual stomach in patients all with previous distal subtotal gastrectomy in 1982<sup>[12,13]</sup>, we have treated 85 patients by such approach, and will report it as follows.

## MATERIALS AND METHODS

### Clinical data

There were 74 men and 11 women with age ranging from 34

to 78 years (mean 60.3 years). The interval between the time of subtotal gastrectomy and the time when the patients were diagnosed having carcinoma of esophagus ranged from 7 to 29 years, averaging 15 years. According to the procedures of subtotal gastrectomy, two cases were classified as Billroth I type, 83 cases Billroth II type, including 27 cases undergoing gastrectomy posterior to colon, and 58 cases anterior to colon. The digestive functions in all of the patients were approximately normal. All the patients presented with the symptoms of dysphagia to some degree with only 42.4%(36/85) being able to take semi-fluid diet. The average body weight of the patients was  $47.7 \pm 10.2$  kg. With regard to the classification of Performance Status, 30.6%(26/85) patients belonged to class 0-1, and 69.4%(59/85) to class 2-4.

In terms of the location of the tumor, 4 cases were localized in upper thoracic esophagus, 62 cases in mid thoracic, and 19 cases in lower thoracic. With regard to gross type, 60 patients belonged to medullary type, 15 ulcerative type, six constrictive type, three fungating type and one intraluminal type. All of cases received barium meal examination of gastrointestinal tract to measure the maximal diameter of residual stomach that was from the fundus to the gastrojejunal anastomosis, and no lesion was found in the residual stomach. The diameter ranged from 5 cm to 9.2 cm, mean 7.9 cm. Forty-three cases in this group were further examined by fiber-gastroscopy, which revealed nothing pathological in their residual stomachs.

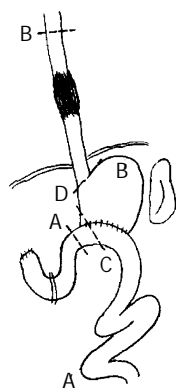
All the patients underwent the resection of involved portion esophagus through left thoracotomy under the intratracheal anesthesia, except for 7 cases being found to have unresectable tumors, in which 5 cases had the local tumors infiltrating the aortic arch or / and bronchus, and two cases were found to be accompanied with metastasis of liver and extensive spread of intra-abdominal cavity.

Seventy-eight cases underwent the resection of the carcinoma of esophagus with a resectability rate of 91.8%(78/85), including 64 cases for radical resection and 14 cases for palliative resection. As for length of the involved segment of esophagus, 21 cases were shorter than 3 cm, 17 cases ranged from 3-5 cm, and 40 cases longer than 5 cm. The maximal diameters of residual stomachs measured from the fundus to the gastrojejunal anastomosis, ranged from 6 cm to 11.3 cm, mean 9.1 cm which was in contrast to X-ray examination. According to the location of anastomosis sites, 5 cases were localized in neck, 51 above aortic arch, and 22 below aortic arch. Sixty-five patients were anastomosed by handwork, 13 by mechanical anastomat, including 10 above aortic arch and 3 below aortic arch. During the operation, a silica-gel catheter with a metal guiding core was inserted into the jejunum through nostril for 30 cases<sup>[14]</sup>. Those patients were fed with mixed milk or nutritional fluid through the nasal feeding catheter one day after operation. The patients had a meal 5-10 d following operation (mean 7 d). The span of hospitalization ranged from 14 to 52 d (mean 14.6 d).

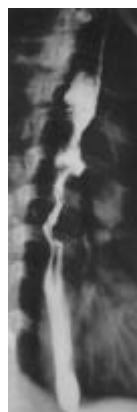
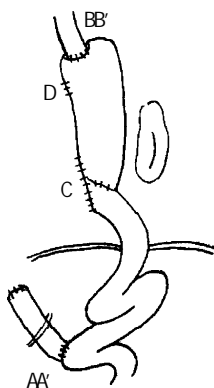
### Procedure of operation

Our approach was through a left thoracotomy incision. The tumor was examined and if it appeared to be resectable, the left diaphragm was opened and the exploration of abdominal

cavity was done. After examining the operative condition of previous subtotal gastrectomy, the spleen, splenic hilus and pancreatic tail were dissociated from the back of peritoneum. Meanwhile, two to four short gastric blood vessels should be preserved to provide adequate blood supply for the residual stomach. The residual stomach, afferent and efferent loops of jejunum were mobilized from peripheral cohesion, and the previous gastrojejunal anastomosis was brought into the left thorax, the afferent loop of jejunum close to gastrojejunal anastomosis was transected and the gastric end was closed. If the part of gastrojejunal anastomosis was removed at the same time, the length of the residual stomach may be prolonged (Figure 1: C). The afferent loop is rejoined to the efferent loop 30 cm below the original gastrojejunostomy (Roux-en-Y method, Figure 2: AA'). The cardia was transected and closed (if mechanical approach was used, the cardia was not closed temporarily, Figure 1: D). The residual stomach, gastrojejunal anastomosis, efferent of jejunum, spleen, tail of pancreas were all transposed into the left thoracic cavity. The esophagus was mobilized and the upper end of esophagus resected 5 cm above the tumor as described in Sweet's esophago-gastrostomy (Figure 2: BB'). If the staple was used, the esophagus was anastomosed to the fundus of the substitution through the cardiac, and the end of cardiac was shut soon after. The splenic ligament was fixed firmly to the thoracic wall to reduce the tension on the esophagogastric anastomosis. If the anastomosis was performed in neck, the fundus of the residual stomach could be brought up into the neck through thoracic cavity or the bed of esophagus. The approach of the operation is represented diagrammatically in Figures 1 and 2. Preoperative and postoperative barium meal examinations, postoperative roentgenogram of chest and field of operation in one of the patients are shown in Figures 3-7.



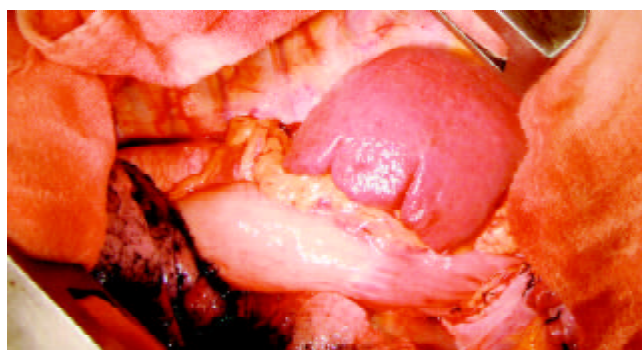
**Figure 1** Resection range. **Figure 2** Postoperative situation.



**Figure 3** Preoperative barium meal examination, showing mid-thoracic esophageal tumor.



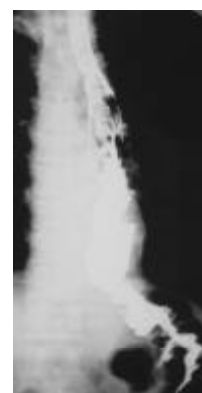
**Figure 4** Preoperative barium meal examination, showing residual stomach.



**Figure 5** Field of operation, showing the spleen in the left thoracic cavity.



**Figure 6** Postoperative roentgenogram of chest, showing the spleen shadow in the left thoracic cavity.



**Figure 7** Postoperative barium meal examination, showing the esophagogastric anastomosis above the aortic arch, the residual stomach with previous gastrointestinal anastomosis, and the Roux-en-Y anastomosis.

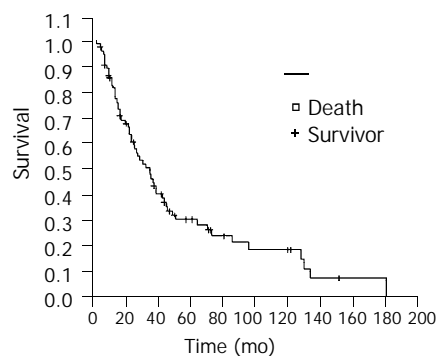
### Follow-up and statistical analysis

All the patients were followed up after operation. The patients failed to be followed up were deemed as death according to the last time they could be contacted. The survival rate was calculated on the basis of Kaplan-Meier procedure by statistical software SPSS10.0. The condition of eating, the body weight and the Performance Status for the patients recruited in our study 3 months after the operation. Enumeration data and measurement data were analyzed by  $\chi^2$  and  $t$  test, respectively.

### RESULTS

Seventy-eight patients received resection of the tumor, including 24 cases having grade I of squamous carcinoma, 47 cases having grade II of squamous carcinoma, 4 cases grade III of squamous carcinoma, 2 cases adenocarcinoma and 1 case

carcinosarcoma. There were two cases having positive resection margins. With regard to lymph node status, 34 patients were complicated with metastasis of lymph node, and 43 patients without. According to UICC1997 PTNM stage, 5 patients were classified as stage I, 30 stage II, and 43 stage III. The complication rate following operation was 10.3%(8/78), with two cases suffering from arrhythmia, two cases having pneumonia and four cases having anastomotic leakage. All these patients with complications were cured through conservative management without operation death. The follow-up rate was 91.0%(71/78), six patients lost contact. The 1, 3, 5, and 10yr survival rate were 85.7%, 50.7%, 30.6% and 18.8%, respectively (Figure 8). The patients undergoing resection could take semi-fluid diet, accounting for 92.3%(72/78). Among all the patients, 84.7%(72/85) could have 300±500 mL volume of semi-fluid diet. The mean body mass was 49.8±8.9 kg. Compared with the previous body mass, 41.0%(32/78) patients got a body weight gain after operation. In term of the grade of Performance Status, 72.9%(62/85) patients had grade 0-1, and 27.1%(23/85) grades 2-4. Apart from the body mass, the difference between the index of quality of life before operation and the comparative index after operation was statistically significant ( $P<0.05$ ).



**Figure 8** Kaplan-Meier survival curve.

## DISCUSSION

Cancer of esophagus in Chaoshan region is prevalent with a high incidence, so are the gastroduodenal diseases, especially the peptic ulcer. Therefore, there are some patients with esophageal carcinoma after subtotal gastrectomy in this area. The incidence rate of these patients was 2.3%(85/3632) compared with the patients with esophageal carcinoma treated surgically in our hospital in the same period, which demonstrates the management of these patients should also be emphasized. The average interval between gastrectomy and diagnosis of esophageal carcinoma was 15 years. The mean age of the patients was 60.3 years, with no significant difference compared with other patients suffering from esophageal carcinoma without subtotal gastrectomy. So it may be suggested the development of carcinoma of esophagus is not obviously correlated with the history of surgical operation of stomach. The relationship between esophageal carcinoma and previous gastrectomy for benign ulcer disease are still not clear<sup>[9,10,15-19]</sup>.

As for the influence of previous operation, the colon or jejunum is the most popular choice as a substitution in such patients. This approach is complicated and can greatly affect the postoperative quality of life<sup>[13,20,21]</sup>. For this reason, some patients were not accessible to resection, but were treated by radiotherapy. When the demand of the resection of esophageal carcinoma was met, the procedure of using the residual stomach to reconstruct the digestive tract, is the best choice, which not only facilitates the recovery of digestive function following operation, but also improves the quality of life in patients with

esophageal carcinoma after previous subtotal gastrectomy. In the process of this procedure, the residual stomach must have sufficient length for lifting, and good blood supply. The blood supply of the residual stomach originated from the short artery. Transferring spleen, tail of pancreas to thoracic cavity can provide the residual stomach with good blood supply, and benefit the lifting length of it. Amputating at the jejunal afferent loop near anastomosis, and making the jejunal Roux-Y anastomosis, fully dissociating the previous operative cohesion could also prolong the lifting length and decrease the lifting tension of the residual stomach. After being fully dissociated, the residual stomach can be lifted up by about 20 cm. Four cases were complicated with anastomotic leakage in the group, including 3 cases with thoracic leakage<sup>[13]</sup> occurring during the early application of this technique. The causes of the thoracic leakage might be related to the local infection and suturing skill. The cause of the other case with anastomotic leakage in neck probably was connected with the tension of the residual stomach. Recently, we replaced manual manipulation with mechanical anastomosis, using staple to close the cardiac and the lesser curvature of stomach. This is better for the extension of the residual stomach, reducing the traction and tension of anastomosis. At the early period after operation, the patients were offered nasal feeding nutrition, which was conducive to the rehabilitation and reduction of complications for patients after resection.

So far, there has been no clear report on the benefit of this procedure, which preserves the residual stomach and anastomoses it to esophagus, in terms of the long-term effect and quality of life for the patients with esophageal carcinoma after subtotal gastrectomy<sup>[21-29]</sup>. Compared with the management of the other patients with esophageal carcinoma in corresponding period, the difference between the means of operation and the findings of pathology in this group was not significant. The 5- and 10-year survival rates were 30.6% and 18.8% respectively. In comparison of the long-term survival rate of our group and those of the other big groups<sup>[10,30-33]</sup>, no significant difference could be found. All these demonstrate that such an approach can achieve a good efficacy. The patients were satisfied with their quality of life, and the majority of them could have semi-fluid diet. Over 50% of them had a body mass gain. The grade of Performance Status of them also improved greatly. In conclusion, the above evidences further confirm that in the management of the patients with carcinoma of esophagus after previous subtotal gastrectomy, the technique, which used the residual stomach to reconstruct the alimentary tract, not only accomplished a rather good long-term survival rate, but also improved the quality of patients' life. So it is a simple operative approach with few trauma and good results.

## REFERENCES

- 1 **Xie ZL**, Zhu KS, Liu SY, Zhang LD. Surgical Treatment of esophageal carcinoma in gastrectomized patients. *Zhongguo Zhongliu Linchuang* 1998; **25**: 570-572
- 2 **Zhang YM**, Chi QM, Li ZM, Zhang BJ, Yang RS, Chen JC, Zhang LM, Li DT, Gao HJ. Surgical Treatment of esophageal carcinoma and cardiac carcinoma after gastrectomy: a report of 24 cases. *Zhongliu Fangzhi Zazhi* 1999; **6**: 188-189
- 3 **Wei JY**, Chen BJ, Sun M. Surgical mode of esophageal and cardiac carcinoma after gastrectomy. *Jiangshu Yiyao* 1999; **25**: 742-744
- 4 **Fu CG**, Gao HC, Cai YJ, Kang JR. Surgical Treatment of esophageal and cardiac cancer after gastrectomy: report of 22 cases. *Fujian Yiyao Zazhi* 2000; **22**: 10-11
- 5 **Jia P**, Lin HX. Reconstruction of esophagus with colon by anterior sterna for esophageal cancer after gastrectomy: report of 12 cases. *Xiandai Zhenduan Yu Zhiliao* 1998; **9**: 295
- 6 **Xu ZF**, Sun YC, Da ZW, Zhao XW, Chen HQ. Surgical Treatment of primary cardiac and esophageal carcinoma of remnant

- stomach. *Jiefangjun Yixue Zazhi* 1997; **22**: 167-168
- 7 **Ping YM**, Yan JS, Du XQ. Clinical application and technical problems of colonic interposition for esophageal substitution. *Zhonghua Waikē Zazhi* 1994; **32**: 755-756
  - 8 **Takemura M**, Higashino M, Osugi H, Tokuhara T, Kaseno S, Kinoshita H. Surgical treatment of esophageal cancer in four patients after gastrectomy for gastric cancer. *Nippon Kyobu Geka Gakkai Zasshi* 1996; **44**: 89-94
  - 9 **Mafune K**, Tanaka Y, Ma YY, Takubo K. Synchronous cancers of the esophagus and the ampulla of Vater after distal gastrectomy: successful removal of the esophagus, gastric remnant, duodenum, and pancreatic head. *J Surg Oncol* 1995; **60**: 277-281
  - 10 **Tachibana M**, Abe S, Yoshimura H, Suzuki K, Matsuura H, Nagasue N, Nakamura T. Squamous cell carcinoma of the esophagus after partial gastrectomy. *Dysphagia* 1995; **10**: 49-52
  - 11 **Kato H**, Tachimori Y, Watanabe H. Surgical treatment for thoracic esophageal carcinoma in patients after gastrectomy. *J Surg Oncol* 1992; **51**: 94-99
  - 12 **Lu SJ**, Chen JQ, Chen YQ, Yang JS. Surgical operation of esophageal carcinoma after subtotal gastrectomy: esophagogastrostomy. *Aizheng* 1986; **5**: 41-44
  - 13 **Lu SJ**, Chen BX. Operative technique for carcinoma of the oesophagus after distal subtotal gastrectomy: a new method using the residual stomach to reconstruct the alimentary tract. *Aust N Z J Surg* 1990; **60**: 719-722
  - 14 **Yang JS**, Yang WP, Chen YP, Chen YQ, Yang XH. Clinical application of silica-gel catheter with a metal guiding core in 43 severe patients. *Zhongguo Weizhongbing Jijiu Yixue* 1995; **7**: 242-243
  - 15 **Hsu NY**, Chen CY, Chen JT, Hsu CP. Oesophageal squamous cell carcinoma after gastrectomy for benign ulcer disease. *Scand J Thorac Cardiovasc Surg* 1996; **30**: 29-33
  - 16 **Caygill CP**, Hill MJ, Kirkham JS, Northfield TC. Oesophageal cancer in gastric surgery patients. *Ital J Gastroenterol* 1993; **25**: 168-170
  - 17 **Caygill CP**, Hill MJ. Malignancy following surgery for benign peptic disease: a review. *Ital J Gastroenterol* 1992; **24**: 218-224
  - 18 **Maeta M**, Koga S, Shimizu T, Matsui K. Possible association between gastrectomy and subsequent development of esophageal cancer. *J Surg Oncol* 1990; **44**: 20-24
  - 19 **Lundegardh G**, Adami HO, Helmick C, Zack M. Risk of cancer following partial gastrectomy for benign ulcer disease. *Br J Surg* 1994; **81**: 1164-1167
  - 20 **Deshmane VH**, Sharma S, Shinde SR, Vyas JJ. Functional results following esophagogastrectomy for carcinoma of the esophagus. *J Surg Oncol* 1992; **50**: 153-155
  - 21 **Guo QX**, Xu JH, Lin JQ, Zheng JF. Surgical Treatment of esophageal carcinoma in patients who had had partial gastrectomy: a report of 30 cases. *Zhongguo Zhongliu Linchuang* 1998; **25**: 573-575
  - 22 **Shu ZD**, Guo XY, Tu YR, Li X, Lin M, Wang Y. Surgical Treatment of the esophageal carcinoma after subtotal gastrectomy: (report of 18 cases). *Zhongguo Shiyong Waikē Zazhi* 2000; **20**: 159-160
  - 23 **Xu M**, Zhang SL, Zhou Y. Improving surgical mode for the carcinoma of mid-lower thoracic esophagus after gastrectomy. *Zhonghua Xiongxinxueguan Waikē Zazhi* 1998; **14**: 265
  - 24 **Wu XY**, Zhang XH, Yin FZ, Lu HS, Guan GX. Clinical study of surgical treatment of esophageal cancer after gastrectomy. *Zhongliu* 1998; **18**: 158-160
  - 25 **Chen J**, Cheng KL, He YG. Treatment of esophageal cancer in post-subtotal gastrectomy with esophagogastrostomy. *Linchuang Waikē Zazhi* 1998; **6**: 25-26
  - 26 **Zhang YJ**, Zhang L. Again Operation of esophageal and cardiac cancer after gastrectomy. *Zhonghua Xiongxinxueguan Waikē Zazhi* 1997; **13**: 234-235
  - 27 **Wang JS**, Feng LG, Wang WD, Yang L. Again Operation for cardiac and esophageal carcinoma after gastrectomy. *Tongji Yike Daxue Xuebao* 1994; **23**: 420-422
  - 28 **Song QQ**, Zeng L, Ge D, Chou DH. Surgical Treatment of esophageal and cardiac cancer after gastrectomy. *Shiyong Zhongliu Zazhi* 2001; **16**: 132-133
  - 29 **Huang HR**, Huang Y. Surgical treatment for patients with esophageal cancer after gastrectomy. *Xin Yixue* 2003; **34**: 498-499
  - 30 **Zhang DW**, Cheng GY, Huang GJ, Zhang RG, Liu XY, Mao YS, Wang YG, Chen SJ, Zhang LZ, Wang LJ, Zhang DC, Yang L, Meng PJ, Sun KL. Operable squamous esophageal cancer: current results from the East. *World J Surg* 1994; **18**: 347-354
  - 31 **Shao LF**. Long-term results of surgical resection of early esophageal and cardiac carcinomas. *Zhonghua Waikē Zazhi* 1993; **31**: 131-133
  - 32 **Shao LF**, Chen YH, Gao ZR, Wei GQ, Xu JL, Chen MY, Cheng JH. Surgical treatment of carcinoma of esophagus and gastric cardia: a 34-year Investigation. *Chine Germ J Clinical Oncol* 2002; **1**: 61-63
  - 33 **Huang YT**, Luo YG. Management of esophageal cancer: what progress has been made? *Zhonghua Weichang Waikē Zazhi* 2001; **4**: 133-141

Edited by Ma JY Proofread by Zhu LH

# Antitumor effects of vaccine consisting of dendritic cells pulsed with tumor RNA from gastric cancer

Bing-Ya Liu, Xue-Hua Chen, Qin-Long Gu, Jian-Fang Li, Hao-Ran Yin, Zheng-Gang Zhu, Yan-Zhen Lin

**Bing-Ya Liu, Xue-Hua Chen, Qin-Long Gu, Jian-Fang Li, Hao-Ran Yin, Zheng-Gang Zhu, Yan-Zhen Lin**, Department of Surgery, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Second Medical University, Shanghai 200025, China

**Supported by** National Natural Science Foundation of China, No. 30170915, Health Ministry of China, No. 9802292, and Shanghai Medical Development Foundation from the Health Bureau of Shanghai, No.983008

**Correspondence to:** Bing-Ya Liu, Ph.D., Department of Surgery, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Second Medical University, Shanghai 200025, China. digsurgliu@netscape.net  
**Telephone:** +86-21-64674654 **Fax:** +86-21-64373909

**Received:** 2003-08-23 **Accepted:** 2003-10-07

## Abstract

**AIM:** To investigate the immunotherapeutic potential of vaccine consisting of dendritic cells (DCs) pulsed with total RNA from MFC gastric cancer cells.

**METHODS:** DCs were prepared from the spleens of strain 615 mice by magnetic cell sorting (MACS). After culture for 24 h, DCs were pulsed with total RNA from MFC gastric cancer cells. Mice of one group were immunized with tumor RNA pulsed DC (RNA/DC) at the dosage of  $1 \times 10^6$  on d 14 and 7 by s.c. inoculation before tumor implantation. Mice of another group were immunized with unpulsed DC (UDC) at the same dosage on days as the RNA/DC group. The third group of control mice was untreated. On d 0, all the mice were challenged with s.c. injections of  $5 \times 10^5$  MFC gastric cancer cells. After inoculation, the mice were monitored closely with respect to tumor growth. Activities of NK cells in PBL and splenocytes and CTL were tested.

**RESULTS:** On d 21 after tumor cell inoculation, the mice of control group manifested the largest tumors with volume at a mean of  $2.6323 \pm 1.1435 \text{ cm}^3$ , followed by the UDC and RNA/DC groups with mean volumes at  $0.7536 \pm 0.3659 \text{ cm}^3$  and  $0.3688 \pm 0.6571 \text{ cm}^3$ , respectively. The activities of NK cells in PBL and splenocytes in RNA/DC group were 66.2% and 65.4%, respectively, higher than that in the control group. The tumor specific CTL activity in RNA/DC group was 49.5%, higher than that in the control group.

**CONCLUSION:** The tumor vaccine with DCs pulsed with total RNA from gastric cancer cells possesses the ability to stimulate tumor specific CTL activity and to establish anti-tumor immunity when administered *in vivo*.

Liu BY, Chen XH, Gu QL, Li JF, Yin HR, Zhu ZG, Lin YZ. Antitumor effects of vaccine consisting of dendritic cells pulsed with tumor RNA from gastric cancer. *World J Gastroenterol* 2004; 10(5): 630-633

<http://www.wjgnet.com/1007-9327/10/630.asp>

## INTRODUCTION

T cells, in particular CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs),

are regarded as the principal effectors of anti-tumor immunity. Several studies have demonstrated that tumor-specific CTL can be induced to recognize peptide epitopes presented on the tumor cell surface in the context of MHC class I molecules. Tumors, however, have evolved various mechanisms to escape from host immune surveillance, such as loss of class I or antigen variants, secretion of immuno-suppressive agents, or development of antigen-specific T cell clonally anergy due to lack of co-stimulation<sup>[1]</sup>.

The dendritic cell (DC) system of antigen-presenting cells (APCs), is the initiator and modulator of immune response<sup>[2]</sup>. DCs are efficient stimulators of B and T lymphocytes. DCs have been known to be highly specialized antigen-presenting cells and principal activators of resting or naive T cells *in vitro* and *in vivo*, capable of efficiently transporting antigens<sup>[3]</sup>.

Immature DCs are capable of capturing and processing antigens extensively, they are also able to internalize apoptotic cells by binding to them via adhesion molecules<sup>[4]</sup>. After the uptake and processing of antigens, DCs leave peripheral tissues and migrate to lymphoid organs in order to present antigens to T lymphocytes. In lymph nodes, DCs form clusters with T lymphocytes by means of various adhesion molecules, in particular CD54(ICAM-1), CD102(ICAM-3) and CD58 (LFA-3), that are highly expressed by activated DCs<sup>[5]</sup>. The DC/T cell interaction is stabilized by the specific ligation of T cell receptors (TCR) of the MHC-peptide complex, which delivers the first activation signal to T lymphocytes. The second costimulatory signal is absolutely necessary to allow T cell activation and proliferation. This signal is mediated by the interaction of CD80(B7-1) and CD86(B7-2), both of which present on DC, with T cell CD28. Expression of a large amount of MHC costimulatory molecules and production of IL-12 by mature DCs turn them into very potent professional APCs<sup>[6]</sup>. DCs pulsed with tumor antigen can induce specific CTL activity<sup>[7]</sup>.

The aim of the present study was to investigate the ability of DC pulsed with tumor RNA to stimulate specific CTL reaction and the potential usefulness of DC-based vaccines for the treatment of cancer patients.

## MATERIALS AND METHODS

### *Mice and cell line*

Seven to eight wk-old strain 615 mice (H-2K<sup>k</sup>) used in these experiments, were obtained from the Chinese Academy of Medical Sciences (Beijing, China). The murine gastric carcinoma cell line MFC derived from strain 615 mouse was provided by the Chinese Academy of Medical Sciences. MFC was cultured in MPRI-1640 medium containing 100 g/L FCS.

### *Isolation of dendritic cells from mouse spleen*

Spleen cells were prepared and treated with collagenase D. Splenocytes were labeled with mouse CD11c<sup>+</sup> DC MicroBeads (MACS, Miltenyi Biotec) according to the manufacturer's manual. In brief,  $10^8$  splenocytes were resuspended in 400  $\mu\text{L}$  buffer and 100  $\mu\text{L}$  of MACS CD11c MicroBeads was added. After thoroughly mixed, the splenocytes were incubated for

15 min at 6–12 °C, followed by a wash with 10–20× labeling volume of buffer. Cells were resuspended in 500 µL of buffer and applied onto the MS<sup>+</sup>/MS<sup>+</sup> column to allow removal of the negative cells. Three rinses with 500 µL of buffer were performed prior to removal of the column from the separator. The column was placed in a collection tube, and 1 ml of buffer was flushed into the column to secure the positive cells. The positive cells were cultured in IMDM containing 200 mL/L FCS, 100 ng/mL IL-4, 100 ng/mL GM-CSF, 10 ng/mL TNF-α for 24 h, then examined by scanning electron microscope and flow cytometry.

#### Preparation of tumor total RNA

Tumor total RNA was isolated from actively growing tissue culture cells, 1×10<sup>7</sup> MFC murine gastric cancer cells were lysed in 1 mL of Trizol reagent (Life Technologies) prepared according to the protocol provided by the manufacturer. One mL of Trizol reagent was added to 1×10<sup>7</sup> of MFC cells which were well mixed well prior to incubation for 5 min at room temperature. Then 0.2 mL of chloroform was added and the mixture was thoroughly mixed and incubated for 3 min at room temperature. After centrifugation of 12 000 g for 15 min at 4 °C, contents were transferred in the aqueous phase to a fresh tube, then 500 µL of isopropyl alcohol was added and mixed well, followed by incubation at room temperature for 10 min and centrifuged at 12 000 g for 10 min at 4 °C. The RNA pellet was washed with 1 mL of 750 mL/L ethanol, and redissolved in RNase free water.

#### Pulsing DCs with tumor RNA

The procedure used for pulsing DCs with tumor RNA was described by Boszkowski<sup>[8]</sup>. In brief, DCs were washed twice in Opti-MEM medium (GIBCO BRL). Cells were resuspended in Opti-MEM medium at 2–5×10<sup>6</sup> cells/mL and transferred into 15 mL polypropylene tubes (Falcon). The cationic lipid, DOTAP (Boehringer Mannheim), was used to deliver RNA into the cells, and 25 µg of total RNA in 500 µL Opti-MEM and 50 µg of DOTAP in 500 µL Opti-MEM were mixed in 12×75 mm polystyrene tubes at room temperature for 20 min. The complex was added to the DCs and incubated at 37 °C for 2–4 h.

#### Immunization of mice using tumor RNA pulsed DCs vaccine and tumor challenge

Tumor RNA pulsed DCs (RNA/DC) and unpulsed DCs (UDC) were collected, washed twice with PBS and then resuspended in PBS. Mice from the respective treatment groups were immunized with these preparations. Each mouse received the designated dose of 1×10<sup>6</sup> DCs s.c. with an intervening period of 7 d before administration of the second dose. The mice untreated were used as control. Twelve mice were in RNA/DC group, 10 mice in UDC group, and 12 mice in control group.

Seven days after vaccination with DCs for the second and last time, each mouse in the RNA/DC group received inoculation of 5×10<sup>5</sup> MFC stomach cancer cells subcutaneously. The animals were closely monitored until the first palpable tumor appeared. Thereafter, two-dimensional tumor measurements were made using calipers, the measurements were recorded every 3–4 d.

#### Cytotoxicity and cytokine analysis

Three weeks after tumor challenge, all the mice were sacrificed. Their spleens were removed and placed in PBS. The splenocytes were depleted of red blood cells through incubation in 8.4 g/L ammonium chloride for 10 min at 37 °C. The samples were then washed twice with PBS. The cells obtained were resuspended with RPMI-1640 containing 100 g/L FBC, 2β-

mercaptoethanol and 10 u/mL IL-2. Splenocytes were stimulated with irradiated MFC gastric cancer cells (5 000 rads) in the ratio of 10:1 (E:T). The samples were then cultured for 5 days and CTLs from each culture were tested. Cell mediated lysis was confirmed *in vitro* using standard chromium (<sup>51</sup>Cr)-release assay.

When the mice were sacrificed, p40 subunits of IL-12 in serum were measured using standard ELISA (R & D).

## RESULTS

### DCs phenotype

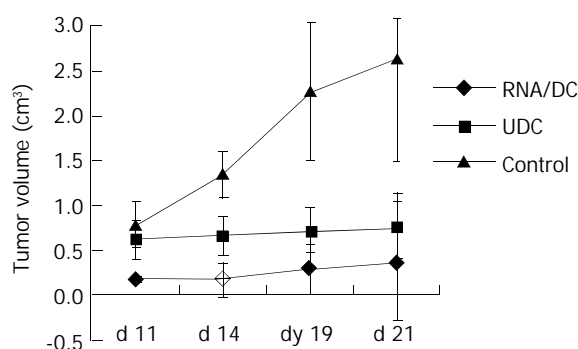
DCs were cultured in IMDM containing 200 g/L FCS, 100 ng/mL IL-4, 100 ng/mL GM-CSF, 10 ng/mL TNF-α for 24 h after sorted by mouse CD11C<sup>+</sup> DC MicroBeads. DCs phenotypes were detected by flow cytometry with H-2KK, I-EK, CD80, and CD 86 (Table 1).

**Table 1** DCs phenotypes before and after culture

	Before culture (%)	After culture (%)
H-2K <sup>K</sup>	92.1	91.6
I-E <sup>K</sup>	28.1	59.2
CD80	21.0	76.4
CD86	23.1	61.3

### Induction of host protective immunity against tumor by RNA pulsed DCs

To determine whether RNA pulsed DC could induce host protective immunity against MFC gastric cancer, strain 615 mice were immunized subcutaneously with RNA pulsed DCs or unpulsed DCs. Seven days after immunization, the mice were inoculated subcutaneously with 5×10<sup>5</sup> MFC murine gastric cancer cells. The results (Figure 1) showed that the mice in the control group (control mice) were all tumor positive on d 7, while the mice immunized with RNA pulsed DCs demonstrated a significant delay in tumor development (7 vs 11 d). RNA pulsed DC immunization reduced tumor incidence significantly. In the RNA/DC group, 41.7% of the mice (5/12) were free from tumor; in the UDC group, 80% of the mice (8/10) developed tumors, and all the mice (12/12) developed tumor in control group on 21 d after tumor cell inoculation. On d 21 after tumor cell inoculation, the tumor volume of the control group attained a mean of 2.6323±1.1435 cm<sup>3</sup>, followed by the UDC and RNA/DC groups with mean volumes of 0.7536±0.3659 cm<sup>3</sup> and 0.3688±0.6571 cm<sup>3</sup>, respectively (Figure 1).

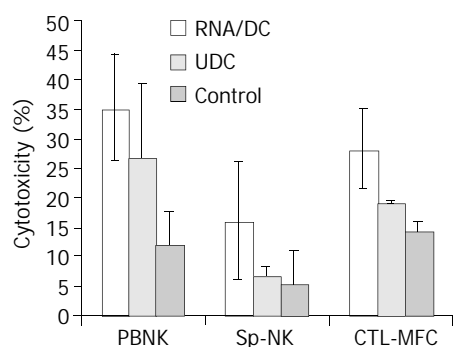


**Figure 1** Tumor growth curve. Control group mice (n=12) were inoculated s.c. with 5×10<sup>5</sup> MFC gastric cancer cells without any immunization. UDC group mice (n=10) were immunized twice with unpulsed DCs followed by inoculation s.c. with 5×10<sup>5</sup> MFC gastric cancer cells. RNA/DC group mice (n=12) were immunized twice with RNA pulsed DCs followed by inoculation s.c. with 5×10<sup>5</sup> MFC gastric cancer cells.



### Induction of tumor specific CTL cytotoxicity by RNA pulsed DCs

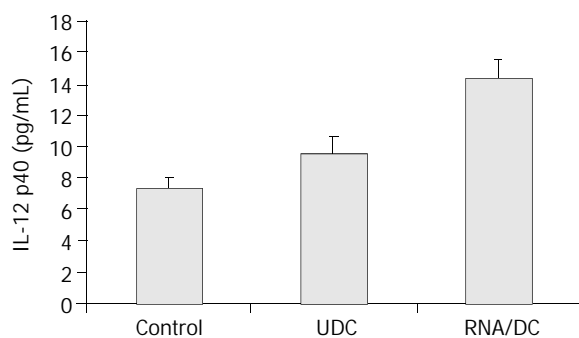
Four weeks after immunization, splenocytes were restimulated *in vitro* with irradiated MFC murine gastric cancer cells for cytotoxic T lymphocyte (CTL) propagation. The results demonstrated that tumor RNA pulsed DCs induced strong tumor specific CTL production. Immunization with MFC tumor RNA pulsed DCs induced stronger lysis of MFC (Figure 2). NK cell activity in PBL or in splenocytes was examined after the mice were sacrificed, revealing that the greatest NK cell activity was seen in the RNA/DC group.



**Figure 2** Cytotoxicity of NK cells and CTL. PBNK: NK cell activity in PBL, Sp-NK: NK cell activity in splenocytes, CTL-MFC: CTL activity to lyses MFC murine gastric cancer cells.

### Serum IL-12 level

Three weeks after tumor challenge, IL-12 in serum was detected by ELISA. The results showed that the serum IL-12 level was the highest in the RNA/DC group among the three groups (Figure 3).



**Figure 3** Serum IL-12 p40 levels in three groups of mice three weeks after tumor challenge.

### DISCUSSION

In recent years, DCs have been found to play an important role in the rejection of tumors by the immune system. Their infiltration of tumors has been associated with an improved prognosis for many neoplasms<sup>[9,10]</sup>.

A number of tumor antigens recognized by CD8+ CTL have been identified<sup>[11]</sup>. These defined antigens could simply be added as 9-11 mer HLA-I-restricted peptides to mature DCs<sup>[12]</sup>. So, the use of peptide-pulsed DC can obtain clearly effective results, but it requires prior knowledge of patients' HLA types and the sequence of the relevant peptide epitopes. It has been suggested that the use of longer peptides or whole proteins, which are not directly present but are endocytosed and processed, is a better way to generate a fully competent DC vaccine since it allows selection by the APC of the optimal 9-mer peptide for presentation<sup>[13]</sup>. After cloning, these tumor

antigens can also be used as cDNA in appropriate vectors to load DCs. But there are three major restrictions when defined tumor antigen is used. First, effective TSA or TAA is not identified in a large number of cancers, especially in gastric cancer. Second, it is clear that immunotherapy approaches directing against a unique antigen favor the selection of a tumor mutant that has selectively lost its ability to present efficiently to the defined antigen<sup>[14]</sup>. Third, the acquisition of antigen-specific CTL *in vitro* is not a guarantee of the development of a curative immune response *in vivo*.

To bypass these disadvantages, several alternative methodologies have been developed. Unfractionated MHC-I-presented peptides could be eluted from tumor cells and loaded onto DC<sup>[11]</sup>. DCs could also be pulsed with whole tumor antigens, such as cell lysates<sup>[15]</sup>, cell extracts<sup>[16]</sup>, apoptotic cells<sup>[4,17]</sup>, total RNA or mRNA<sup>[7]</sup>. The method of fusing tumor cells with DCs has also been explored<sup>[18,19]</sup>. The recent demonstration of cell-contact generated by short-term coculture of DC with tumor cells without cell fusion could result in a potent immunogen and was particularly relevant<sup>[20]</sup>. DCs pulsed with tumor RNA may thus be a potential effective means to induce host T cell-mediated anti-tumor responses. Boczkowski *et al.* have recently demonstrated that vaccination with tumor RNA pulsed DCs could elicit T cell protective immunity against tumor challenge and induce immune rejection of established tumors<sup>[8]</sup>.

Gastric cancer is one of the tumors with very high heterogeneity. Host immune status in patients with gastric cancer is usually poor. Up to the present, immunotherapy for gastric cancer has not been clearly effective. The use of DC-based tumor vaccine can provide a glimmer of hope for patients with gastric cancer. In this experiment, we used tumor total RNA as tumor whole antigen to pulse DCs. All the antigens were processed and selectively presented to T cells by DCs. The results following the use of tumor total RNA pulsed DCs as vaccine, indeed, induced anti-tumor immune responses by stimulating NK cells and tumor-specific CTL activity in the mouse.

For clinical trials of DC-based vaccine for cancer, other matters must be considered. The first is the source of DCs for clinical use. The second is the choice of tumor antigens and method for antigen loading of DCs. Third, the DC dose-response relationship should be determined using individual cell preparation methods. Finally, the optimal route and frequency of administration need to be determined.

### ACKNOWLEDGMENT

We want to thank Dr. Dong-Qing Zhang, Shanghai Institute of Immunology for his good advice, and Yi Zhang, research assistant, for her work and Dr. Ming-Jun Zhang for his help in animal experimentation.

### REFERENCES

- 1 Zitzvogel L, Mayordomo JJ, Tjandrawan T, Deleo AB, Clarke MR, Lotze MT, Storkus WJ. Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J Exp Med* 1996; **183**: 87-97
- 2 Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; **392**: 245-252
- 3 Constant S, Sant' Angelo D, Pasqualini T, Taylor T, Levin D, Flavell R, Bottomly K. Peptide and protein antigens require distinct antigen-presenting cell subsets for the priming of CD4+ T cells. *J Immunol* 1995; **154**: 4915-4923
- 4 Albert ML, Pearce SF, Francisco LM, Sauter B, Roy P, Silverstein RL, Bhardwaj N. Immature dendritic cells phagocytose apoptotic cells via alpha5beta1 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J Exp Med* 1998; **188**: 1359-1368

- 5 **Scheeren RA**, Koopman G, Van der Baan S, Meijer CJ, Pals ST. Adhesion receptors involved in clustering of blood dendritic cells and T lymphocytes. *Eur J Immunol* 1991; **21**: 1101-1105
- 6 **Tarte K**, Klein B. Dendritic cell-based vaccine: a promising approach for cancer immunotherapy. *Leukemia* 1999; **13**: 653-663
- 7 **Zhang JK**, Li J, Chen HB, Sun JL, Qu YJ, Lu JJ. Antitumor activities of human dendritic cells derived from peripheral and cord blood. *World J Gastroenterol* 2002; **8**: 87-90
- 8 **Boczkowski D**, Nair SK, Snyder D, Gilboa E. Dendritic cells pulsed with RNA are potent antigen-presenting cells *in vitro* and *in vivo*. *J Exp Med* 1996; **184**: 465-472
- 9 **Ishigami S**, Aikou T, Natsugoe S, Hokita S, Iwashige H, Tokushige M, Sonoda S. Prognostic value of HLA-DR expression and dendritic cell infiltration in gastric cancer. *Oncology* 1998; **55**: 65-69
- 10 **Saito H**, Tsujitani S, Ikeguchi M, Maeta M, Kaibara N. Relationship between the expression of vascular endothelial growth factor and the density of dendritic cells in gastric adenocarcinoma tissue. *Br J Cancer* 1998; **78**: 1573-1577
- 11 **Boon T**, Van der Bruggen P. Human tumor antigens recognized by T lymphocytes. *J Exp Med* 1996; **183**: 725-729
- 12 **Tsai V**, Southwood S, Sidney J, Sakaguchi K, Kawakami Y, Appella E, Sette A, Celis E. Identification of subdominant CTL epitopes of the GP100 melanoma-associated tumor antigen by primary *in vitro* immunization with peptide-pulsed dendritic cells. *J Immunol* 1997; **158**: 1796-1802
- 13 **Nieda M**, Nicol A, Kikuchi A, Kashiwase K, Taylor K, Suzuki K, Tadokoro K, Juji T. Dendritic cells stimulate the expansion of bcr-abl specific CD8<sup>+</sup> T cells with cytotoxic activity against leukemic cells from patients with chronic myeloid leukemia. *Blood* 1998; **91**: 977-983
- 14 **Ikeda H**, Lethe B, Lehmann F, van Baren N, Baurain JF, de Smet C, Chambost H, Vitale M, Moretta A, Boon T, Coulie PG. Characterization of an antigen that is recognized on a melanoma showing partial HLA loss by CTL expressing an NK inhibitory receptor. *Immunity* 1997; **6**: 199-208
- 15 **Tang ZH**, Qiu WH, Wu GS, Yang XP, Zou SQ, Qiu FZ. The immunotherapeutic effect of dendritic cells vaccine modified with interleukin-18 gene and tumor cell lysate on mice with pancreatic carcinoma. *World J Gastroenterol* 2002; **8**: 908-912
- 16 **Asavaroengchai W**, Kotera Y, Mule JJ. Tumor lysate-pulsed dendritic cells can elicit an effective antitumor immune response during early lymphoid recovery. *Proc Natl Acad Sci U S A* 2002; **99**: 931-936
- 17 **Albert ML**, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 1998; **392**: 86-89
- 18 **Zhang JK**, Li J, Zhang J, Chen HB, Chen SB. Antitumor immunopreventive and immunotherapeutic effect in mice induced by hybrid vaccine of dendritic cells and hepatocarcinoma *in vivo*. *World J Gastroenterol* 2003; **9**: 479-484
- 19 **Zhang J**, Zhang JK, Zhou SH, Chen HB. Effect of a cancer vaccine prepared by fusions of hepatocarcinoma cells with dendritic cells. *World J Gastroenterol* 2001; **7**: 690-694
- 20 **Celluzzi CM**, Falo LD Jr. Physical interaction between dendritic cells and tumor cells results in an immunogen that induces protective and therapeutic tumor rejection. *J Immunol* 1998; **160**: 3081-3085

Edited by Wang XL

# Maspin expression and its clinicopathological significance in tumorigenesis and progression of gastric cancer

Meng-Chun Wang, Yan-Min Yang, Xiao-Han Li, Fang Dong, Yan Li

**Meng-Chun Wang, Yan-Min Yang, Yan Li**, Department of Gastroenterology, The Second Affiliated Hospital of China Medical University, Shenyang 110004, Liaoning Province, China  
**Xiao-Han Li, Fang Dong**, Department of Pathology, The Second Affiliated Hospital of China Medical University, Shenyang 110004, Liaoning Province, China

**Correspondence to:** Meng-Chun Wang, Department of Gastroenterology, The Second Affiliated Hospital of China Medical University, Shenyang 110004, Liaoning Province, China. mengchunwang@hotmail.com  
**Telephone:** +86-24-83956947

**Received:** 2003-06-16 **Accepted:** 2003-08-25

## Abstract

**AIM:** To investigate maspin expression in tumorigenesis and progression of gastric cancer and to explore its relevant molecular mechanisms.

**METHODS:** Formalin-fixed and paraffin-embedded tissues from normal mucosa ( $n=182$ ), dysplasia ( $n=69$ ), cancer ( $n=113$ ) of the stomach were studied for maspin expression by immunohistochemistry. Microvessel density (MVD) in gastric cancer was labeled using anti-CD34 antibody. Maspin expression was compared with clinical parameters and MVD of tumors. Caspase-3 expression was also detected in gastric carcinoma by immunohistochemistry. The relationship between Caspase-3 and maspin expression was concerned as well.

**RESULTS:** The positive rates of maspin expression were 79.8% (145/182), 75.4% (52/69) and 50.4% (57/113) in normal mucosa, dysplasia and cancer of the stomach, respectively. Cancer less frequently expressed maspin than normal mucosa and dysplasia ( $P<0.05$ ). Maspin expression showed a significantly negative association with invasive depth, metastasis, Lauren's and Nakamura's classification ( $P<0.05$ ), but not with tumor size, Borrmann's classification, growth pattern or TNM staging ( $P>0.05$ ). The positive rate of Caspase-3 was significantly lower in gastric cancer than in normal gastric mucosa ( $P<0.05$ , 32.7% vs 50.4%). It was noteworthy that maspin expression was negatively correlated with MVD, but positively correlated with expression of Caspase-3 in gastric cancer ( $P<0.05$ ).

**CONCLUSION:** Down-regulated maspin expression is a late molecular event in gastric carcinogenesis. Reduced expression of maspin contributes to progression of gastric cancer probably by inhibiting cell adhesion, enhancing cell mobility, decreasing cell apoptosis and facilitating angiogenesis. Additionally altered expression of maspin underlies the molecular mechanism of differentiation of gastric cancer and supports the different histogenetic pathways of intestinal and diffuse gastric cancers. Maspin expression can be considered as an effective and objective marker to reveal biological behaviors of gastric cancer.

Wang MC, Yang YM, Li XH, Dong F, Li Y. Maspin expression and its clinicopathological significance in tumorigenesis and

progression of gastric cancer. *World J Gastroenterol* 2004; 10(5): 634-637

<http://www.wjgnet.com/1007-9327/10/634.asp>

## INTRODUCTION

Mammary serine protease inhibitor (maspin) was identified by subtractive hybridization as a candidate tumor suppressor protein in normal mammary epithelial cells<sup>[1]</sup>. Maspin gene maps to human chromosome 18q21.3-q23, whose cDNA consists of 2 584 nucleotides encoding for a 42 ku peptide<sup>[2]</sup>. A number of findings support its inhibitory effects on tumors. Levels of maspin expression showed an inverse correlation with progression of malignancies. Mammary carcinoma cells transfected with maspin showed a reduction of tumor growth and metastasis in nude mice, the addition of recombinant maspin decreased the migration potential of breast and prostate cancer cells across a reconstituted basement membrane. More recently, maspin has been shown to inhibit angiogenesis by blocking *in vitro* migration of vascular endothelial cells and by *in vivo* inhibition of rat cornea neovascularization<sup>[1-8]</sup>. It has been documented that maspin transgene expression in mouse mammary gland inhibited Simian virus 40 (SV40) large T-antigen induced breast carcinogenesis and was correlated with increased apoptosis of mammary gland cells<sup>[9]</sup>.

Gastric cancer is one of the commonest malignancies in China, and even in the world. However, the molecular aspects of carcinogenesis and progression of gastric cancer remain elusive<sup>[10-19]</sup>. The purposes of this study were to examine maspin expression in normal gastric mucosa, gastric dysplasia, gastric cancer, and to compare its expression with clinicopathological features of gastric cancer, and to analyze the correlation of maspin expression with Caspase-3 expression and MVD in gastric cancer.

## MATERIALS AND METHODS

### Patients and samples

Normal mucosa ( $n=182$ ), dysplasia ( $n=69$ ), cancer ( $n=113$ ) of the stomach were collected from the Second Affiliated Hospital of China Medical University between Sept. 1996 and Feb. 2002. All tissues were fixed in 40 g/L formaldehyde, embedded in paraffin and incised into 4  $\mu$ m sections. These sections were stained by hematoxylin-and-eosin method to confirm their histological diagnosis and other microscopic characteristics. All patients did not undergo chemotherapy and radiotherapy before operation.

### Evaluation of clinicopathological parameters of gastric cancer

Clinicopathological staging for each gastric carcinoma was evaluated according to the TNM system. Gross appearance of the tumors was described according to Borrmann's classification. Histomorphological architecture of the tumor samples was expressed according to Lauren's and Nakamura's classifications. Growth patterns of gastric cancer were classified into mass-,

nest-, or diffuse- type. Furthermore, tumor diameter, invasive depth and metastasis were determined.

### Immunohistochemistry

Representative and consecutive sections were studied with streptavidin-biotin-peroxidase immunohistochemistry (SABC kit from Boster Biotech.). Anti-maspin, anti-Caspase-3 and anti-CD34 antibodies were purchased from Novocastra, Zhongshan (China) and DAKO respectively. All procedures were implemented according to the instructions of the product. For negative controls, sections were processed as above but treated with PBS (0.01 mol/L, pH7.4) instead of primary antibodies.

### Evaluation of maspin and Caspase-3 immunostaining

The immunoreactivity to maspin and Caspase-3 was localized in cytoplasm. One hundred cells were selected and counted from 5 representative fields of each section by two independent observers. The positive percentage of counted cells was graded semi-quantitatively into one of the four-tier scoring system: negative(-), 5%; weakly positive (+), 5-25%; moderately positive (++), 25-50%; and strongly positive(+++), 50%.

### Microvessel density counting

CD34 antibodies were distributed in cell membranes and cytoplasm of vascular endothelial cells. Modified Weidner's method was used to calculate the microvessel density, which was described as follows. Microvessels in tumor were considered as hot points in vessel counts. Any brown staining endothelial cell or endothelial cell cluster was regarded as a single, countable microvessel. Observers selected such five areas and counted individual microvessels at 400× magnification (*i.e.* 40× objective lens and 10× ocular lens, 0.1885 mm<sup>2</sup> per field).

### Statistical analysis

Statistical evaluation was performed using *chi*-square test to differentiate the rates between different groups, using Spearman correlation test to analyze the rank data, and using one-way ANOVA to differentiate means of different groups.  $P < 0.05$  was considered as statistically significant. SPSS 10.0 software was employed to analyze all data.

## RESULTS

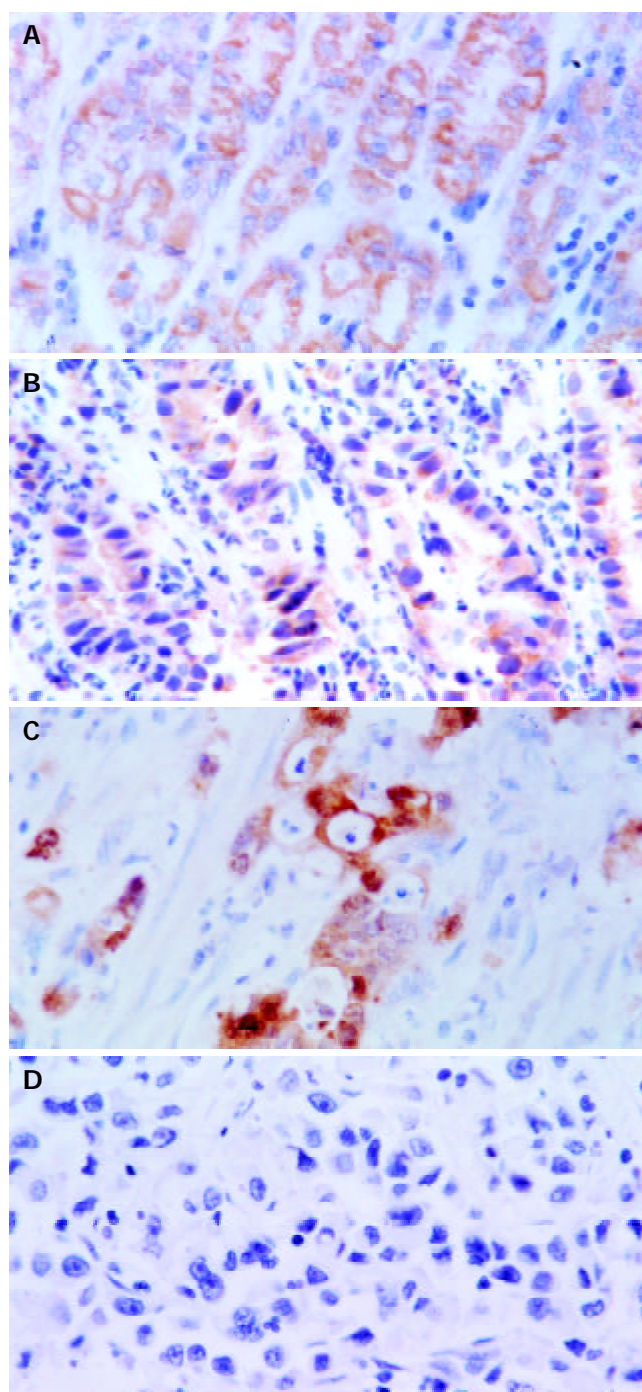
### Maspin expression in normal mucosa, dysplasia, and cancer of the stomach

Figures 1A-D show the positive immunoreactivity to maspin in cytoplasm of epithelial, dysplasia and cancer cells of the stomach. As summarized in Table 1, the positive rates of maspin expression were 79.8% (145/182), 75.4% (52/69), and 50.4% (57/113) in normal mucosa, dysplasia and cancer of gastric cancer, respectively. Normal mucosa and dysplasia more frequently expressed maspin than cancer ( $P < 0.05$ ).

**Table 1** Maspin expression in normal mucosa, dysplasia, and primary cancer of the stomach

Groups	<i>n</i>	Maspin expression		
		-	++	%
Normal mucosa	182	37	145	79.8 <sup>b</sup>
Dysplasia	69	17	52	75.4 <sup>d</sup>
Primary cancer	113	56	57	50.4

Compared with primary cancer <sup>b</sup> $P = 0.000$  ( $\chi^2 = 27.589$ , Pearson'  $r = 0.306$ ), <sup>d</sup> $P = 0.000$  ( $\chi^2 = 11.075$ , Pearson'  $r = 0.247$ ).



**Figure 1** A: Localization of maspin in cytoplasm, and strong expression in gastric epithelial cells (SABC, ×400), B: Strong immunoreactivity of gastric dysplastic cells to maspin (SABC, ×400), C: Strong immunostaining of maspin in gastric papillary adenocarcinoma cells (SABC, ×400), D: Negative expression of maspin in poorly-differentiated gastric adenocarcinoma cells (SABC, ×400).

### Relationship between maspin expression and clinicopathological features of gastric cancer

Table 2 shows that maspin expression had a significantly negative association with invasive depth, metastasis, Lauren's and histological classifications ( $P < 0.05$ ), but not with tumor size, Borrmann's classification, growth pattern or TNM staging ( $P > 0.05$ ).

### Relationship between maspin and Caspase-3 expression, MVD in gastric cancer

The positive rate of Caspase-3 expression was lower in gastric



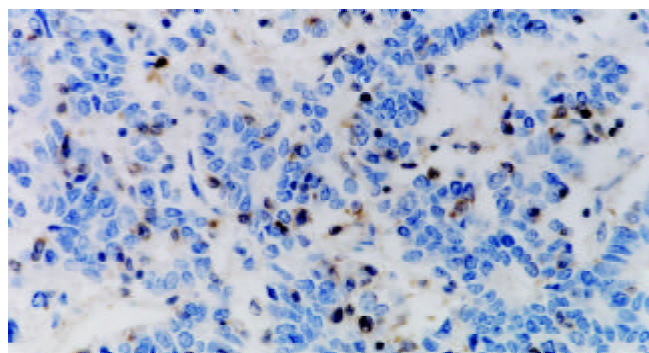
cancer than in normal gastric mucosa ( $P<0.05$ , 32.7% vs 50.4%). It was noticeable that maspin expression was positively correlated with Caspase-3 expression, but negatively with MVD in gastric cancer ( $P<0.05$ ) (Tables 2-3, Figures 2,3).

**Table 2** Relationship between maspin expression and clinicopathological features of gastric cancer

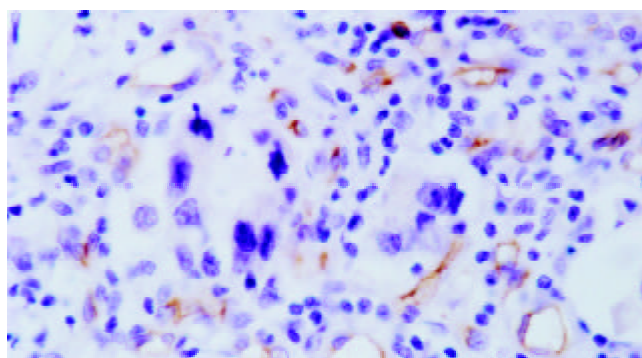
Clinicopathological features	n	Maspin expression					rs	P value
		-	+	++	+++	%		
Tumor size							0.000	0.999
<4 cm	47	22	13	7	5	53.2		
≥4 cm	66	34	11	15	6	48.5		
Borrmann's classification							0.014	0.896
I, II	28	12	9	5	2	57.1		
III, IV	59	30	10	13	6	49.2		
Invasive depth							0.280	0.003
Above submucosa	26	10	4	6	6	61.5		
Muscularis propria	34	12	11	7	4	64.7		
Below subserosa	53	34	9	9	1	35.8		
Metastasis							0.208	0.027
-	75	31	18	19	7	58.7		
+	38	25	6	3	4	34.2		
TNM staging							0.004	0.967
O, I	46	22	11	10	3	52.2		
II, III, IV	67	34	13	12	8	49.3		
Growth pattern							0.032	0.767
Mass	23	13	4	5	1	43.5		
Nest	30	11	6	8	5	63.3		
Diffuse	34	18	9	5	2	47.1		
Lauren's classification							0.228	0.015
Intestinal	36	12	8	11	5	66.7		
Diffuse	57	33	12	8	4	42.1		
Mixed	20	11	4	3	2	45.0		
Nakamura's classification							0.212	0.024
Differentiated	53	20	14	12	7	62.3		
Undifferentiated	60	36	10	1	4	10.0		
Caspase-3 expression							0.246	0.009
-	78	44	17	12	5	56.4		
+~++++	35	12	7	10	6	65.7		

**Table 3** Relationship between maspin expression and MVD in primary gastric cancer

Maspin expression	n	MVD (mean±SD)	F value	P value
-	56	51.72±26.87	4.911	0.029
+~++++	57	41.70±20.92		
Total	113	46.67±24.47		



**Figure 2** Negatively immunostaining of Caspase-3 in poorly-differentiated gastric adenocarcinoma cells and positive expression in infiltrating lymphocytes (SABC, ×400).



**Figure 3** Localization of CD34 antigens in cell membranes and cytoplasm of vascular endothelial cells (SABC, ×400).

## DISCUSSION

Carcinogenesis and progression of malignancies are a complicated multistage process that requires the coordination of multiple genes, including oncogenes and tumor suppressor genes. Genomic instability is one of the driving forces for tumor development. Among all genetic alterations, inactivation of metastasis suppressor genes has been found to be an important factor because of their contribution to malignant change of normal cells and metastasis of tumor cells<sup>[20,21]</sup>.

It is known that maspin is highly expressed in various kinds of normal cells and lowly expressed in cancer cells. Our data showed that maspin expression was gradually reduced from normal mucosa, through dysplasia to cancer of the stomach. The positive rate of maspin expression was lower in gastric cancer than in normal gastric mucosa and gastric dysplasia with no significant difference in the latter two. These results suggested that reduced maspin expression contributed to malignant change of gastric epithelial cells. The down-regulated expression of maspin could be considered as a late molecular event of gastric carcinogenesis. Previous reports indicated that DNA methylation, histone deacetylation or LOH was partially responsible for the silencing of maspin gene expression<sup>[22-24]</sup>. Decreased expression of maspin might be attributable to these genetic alterations in gastric carcinogenesis as mentioned above.

Our study showed that maspin expression was negatively associated with invasion and metastasis of tumors, suggesting its inhibitory effects on progression of gastric cancer. Biliran *et al*<sup>[25]</sup> found that maspin could specifically inhibit cell surface-associated urokinase-type plasminogen activator and fibrinogen-bound tissue-type plasminogen activator, which was correlated with significantly decreased cell invasion potential and motility *in vitro*. Blacque *et al*<sup>[26]</sup> reported that interaction between recombinant maspin and some collagens might contribute to cell adhesion, cell migration and angiogenesis. Seftor *et al*<sup>[27]</sup> demonstrated that maspin was able to regulate integrin expression, indicating that maspin could reduce the invasive phenotype of cancer cells by altering their integrin profile. These findings suggested that maspin expression could inhibit tumor progression *in vivo*, likely through a combination of increased cell adhesion, decreased angiogenesis, and inhibition of tumor cell migration.

Additionally, it was found that undifferentiated gastric carcinomas had a lower expression of maspin as compared with the differentiated ones, suggesting that down-regulated expression of maspin was closely associated with the differentiation of gastric cancer. Diffuse-type gastric cancers had less expression of maspin as compared with the intestinal-type ones. It supported that there were different tumorigenic pathways between diffuse-type and intestinal-type gastric carcinomas. Diffuse-type gastric cancer, the main part of which was undifferentiated

carcinoma, displayed a diffusely invasive growth pattern. It is possible that down-regulation of maspin expression could influence mobility and adhesion of cancer cells.

Our study also showed a negative correlation between maspin expression and MVD in gastric cancer. Song *et al*<sup>[28]</sup> found that maspin-positive colonic adenocarcinomas showed less MVD than maspin-negative ones. Zhang *et al*<sup>[8]</sup> reported that maspin might act directly on vascular endothelial cells to stop their migration towards basic fibroblast growth factor and vascular endothelial growth factor and to limit mitogenesis and tube formation, which could dramatically reduce tumor-associated MVD. These *in vivo* and *in vitro* data suggest that the tumor suppressor activity of maspin may depend in large part on its ability to inhibit angiogenesis and raise the possibility that maspin and similar serpins may be excellent targets for the development of drugs that modulate angiogenesis.

Furthermore, it was found that Caspase-3 expression was increased in gastric cancer with maspin positively expressed. Jiang *et al*<sup>[29]</sup> reported that endogenous maspin expression could enhance staurosporine-induced apoptosis of carcinoma cells as judged by the increased fragmentation of DNA, increased proteolytic inactivation of poly-[ADP-ribose]-polymerase, as well as the increased activation of Caspase-8 and Caspase-3. Li *et al*<sup>[30]</sup> showed that apoptosis induced by manganese-containing superoxide dismutase was associated with elevated maspin expression level. These results indicated that it was possible that maspin expression might inhibit progression of gastric cancer by inducing apoptosis.

In summary, down-regulated maspin expression is a late molecular event in gastric carcinogenesis. Reduced expression of maspin can contribute to progression of gastric cancer by inhibiting cell adhesion, enhancing cell mobility, decreasing cell apoptosis and facilitating angiogenesis. Additionally altered expression of maspin underlies the molecular mechanism of differentiation of gastric cancer and supports the different histogenetic pathways of intestinal and diffuse gastric cancers. Maspin expression can be considered as an effective marker to reveal biological behaviors of gastric cancer.

## REFERENCES

- 1 **Maass N**, Hojo T, Zhang M, Sager R, Jonat W, Nagasaki K. Maspin—a novel protease inhibitor with tumor-suppressing activity in breast cancer. *Acta Oncol* 2000; **39**: 931-934
- 2 **Reis-Filho JS**, Torio B, Albergaria A, Schmitt FC. Maspin expression in normal skin and usual cutaneous carcinomas. *Virchows Arch* 2002; **441**: 551-558
- 3 **Kim DH**, Yoon DS, Dooley WC, Nam ES, Ryu JW, Jung KC, Park HR, Sohn JH, Shin HS, Park YE. Association of maspin expression with the high histological grade and lymphocyte-rich stroma in early-stage breast cancer. *Histopathology* 2003; **42**: 37-42
- 4 **Odero-Marah VA**, Khalkhali-Ellis Z, Schneider GB, Seftor EA, Seftor RE, Koland JG, Hendrix MJ. Tyrosine phosphorylation of maspin in normal mammary epithelia and breast cancer cells. *Biochem Biophys Res Commun* 2002; **295**: 800-805
- 5 **Zou Z**, Zhang W, Young D, Gleave MG, Rennie P, Connell T, Connelly R, Moul J, Srivastava S, Sesterhenn I. Maspin expression profile in human prostate cancer (CaP) and *in vitro* induction of Maspin expression by androgen ablation. *Clin Cancer Res* 2002; **8**: 1172-1177
- 6 **Shi HY**, Liang R, Templeton NS, Zhang M. Inhibition of breast tumor progression by systemic delivery of the maspin gene in a syngeneic tumor model. *Mol Ther* 2002; **5**: 755-761
- 7 **Streuli CH**. Maspin is a tumour suppressor that inhibits breast cancer tumour metastasis *in vivo*. *Breast Cancer Res* 2002; **4**: 137-140
- 8 **Zhang M**, Volpert O, Shi YH, Bouck N. Maspin is an angiogenesis inhibitor. *Nat Med* 2000; **6**: 196-199
- 9 **Zhang M**, Shi Y, Magit D, Furth PA, Sager R. Reduced mammary tumor progression in WAP-TAg/WAP-maspin bitransgenic mice. *Oncogene* 2000; **19**: 6053-6058
- 10 **Yin T**, Ji XL, Shen MS. Relationship between lymph node sinuses with blood and lymphatic metastasis of gastric cancer. *World J Gastroenterol* 2003; **9**: 40-43
- 11 **Yang L**, Kuang LG, Zheng HC, Li JY, Wu DY, Zhang SM, Xin Y. PTEN encoding product: a marker for tumorigenesis and progression of gastric carcinoma. *World J Gastroenterol* 2003; **9**: 35-39
- 12 **Jiang YA**, Zhang YY, Luo HS, Xing SF. Mast cell density and the context of clinicopathological parameters and expression of p185, estrogen receptor, and proliferating cell nuclear antigen in gastric carcinoma. *World J Gastroenterol* 2002; **8**: 1005-1008
- 13 **Zhang H**, Wu J, Meng L, Shou CC. Expression of vascular endothelial growth factor and its receptors KDR and Flt-1 in gastric cancer cells. *World J Gastroenterol* 2002; **8**: 994-998
- 14 **Zhou YN**, Xu CP, Han B, Li M, Qiao L, Fang DC, Yang JM. Expression of E-cadherin and beta-catenin in gastric carcinoma and its correlation with the clinicopathological features and patient survival. *World J Gastroenterol* 2002; **8**: 987-993
- 15 **Fang DC**, Luo YH, Yang SM, Li XA, Ling XL, Fang L. Mutation analysis of APC gene in gastric cancer with microsatellite instability. *World J Gastroenterol* 2002; **8**: 787-791
- 16 **Song ZJ**, Gong P, Wu YE. Relationship between the expression of iNOS, VEGF, tumor angiogenesis and gastric cancer. *World J Gastroenterol* 2002; **8**: 591-595
- 17 **Yao XX**, Yin L, Sun ZC. The expression of hTERT mRNA and cellular immunity in gastric cancer and precancerosis. *World J Gastroenterol* 2002; **8**: 586-590
- 18 **Niu WX**, Qin XY, Liu H, Wang CP. Clinicopathological analysis of patients with gastric cancer in 1200 cases. *World J Gastroenterol* 2001; **7**: 281-284
- 19 **Xin Y**, Li XL, Wang YP, Zhang SM, Zheng HC, Wu DY, Zhang YC. Relationship between phenotypes of cell-function differentiation and pathobiological behavior of gastric carcinomas. *World J Gastroenterol* 2001; **7**: 53-59
- 20 **Jaeger EB**, Samant RS, Rinker-Schaeffer CW. Metastasis suppression in prostate cancer. *Cancer Metastasis Rev* 2001; **20**: 279-286
- 21 **Debies MT**, Welch DR. Genetic basis of human breast cancer metastasis. *J Mammary Gland Biol Neoplasia* 2001; **6**: 441-451
- 22 **Maass N**, Biallek M, Rosel F, Schem C, Ohike N, Zhang M, Jonat W, Nagasaki K. Hypermethylation and histone deacetylation lead to silencing of the maspin gene in human breast cancer. *Biochem Biophys Res Commun* 2002; **297**: 125-128
- 23 **Spring P**, Nakashima T, Frederick M, Henderson Y, Clayman G. Identification and cDNA cloning of headpin, a novel differentially expressed serpin that maps to chromosome 18q. *Biochem Biophys Res Commun* 1999; **264**: 299-304
- 24 **Domann FE**, Rice JC, Hendrix MJ, Futscher BW. Epigenetic silencing of maspin gene expression in human breast cancers. *Int J Cancer* 2000; **85**: 805-810
- 25 **Biliran H Jr**, Sheng S. Pleiotrophic inhibition of pericellular urokinase-type plasminogen activator system by endogenous tumor suppressive maspin. *Cancer Res* 2001; **61**: 8676-8682
- 26 **Blacque OE**, Worrall DM. Evidence for a direct interaction between the tumor suppressor serpin, maspin, and types I and III collagen. *J Biol Chem* 2002; **277**: 10783-10788
- 27 **Seftor RE**, Seftor EA, Sheng S, Pemberton PA, Sager R, Hendrix MJ. Maspin suppresses the invasive phenotype of human breast carcinoma. *Cancer Res* 1998; **58**: 5681-5685
- 28 **Song SY**, Lee SK, Kim DH, Son HJ, Kim HJ, Lim YI, Lee WY, Chun HK, Rhee JC. Expression of maspin in colon cancers: its relationship with p53 expression and microvessel density. *Dig Dis Sci* 2002; **47**: 1831-1835
- 29 **Jiang N**, Meng Y, Zhang S, Mensah-Osman E, Sheng S. Maspin sensitizes breast carcinoma cells to induced apoptosis. *Oncogene* 2002; **21**: 4089-4098
- 30 **Li JJ**, Colburn NH, Oberley LW. Maspin gene expression in tumor suppression induced by overexpressing manganese-containing superoxide dismutase cDNA in human breast cancer cells. *Carcinogenesis* 1998; **19**: 833-839

# Modulation of human telomerase reverse transcriptase in hepatocellular carcinoma

Cheng-Jueng Chen, Satoru Kyo, Yao-Chi Liu, Yeung-Leung Cheng, Chung-Bao Hsieh, De-Chuan Chan, Jyh-Cherng Yu, Horng-Jyh Harn

**Cheng-Jueng Chen, Yao-Chi Liu, Chung-Bao Hsieh, De-Chuan Chan, Jyh-Cherng Yu**, Division of General Surgery, Department of Surgery, Tri-Service General Hospital, Taipei, Taiwan, China  
**Yeung-Leung Cheng**, Division of Thoracic Surgery, Department of Surgery, Tri-Service General Hospital, Taipei, Taiwan, China  
**Satoru Kyo**, Department of Obstetrics and Gynecology, Kanazawa University, Kanazawa, Japan

**Horng-Jyh Harn**, Division of Molecular Medicine, Department of Pathology, Buddhist Tzu-Chi General Hospital, Tzu-Chi University, Hua-Lien, Taiwan, China

**Correspondence to:** Horng-Jyh Harn, MD, PhD, Division of Molecular Medicine, Department of Pathology, Buddhist Tzu-Chi General Hospital, 707, Section 3, Chung-Yang Rd., 970 Hua-Lien, Taiwan, China. duke@tzuchi.com.tw

**Telephone:** +886-2-87927191 **Fax:** +886-2-87927372

**Received:** 2003-10-08 **Accepted:** 2003-12-16

## Abstract

**AIM:** Most cancer cells acquire immortal capability by telomerase activation. The human telomerase reverse transcriptase gene (*hTERT*) is considered to be the major determinant of the enzymatic activity of human telomerase, and the *hTERT* promoter contains several c-Myc binding sites that mediate *hTERT* transcriptional activation. Few studies have examined the role of *hTERT* in hepatocarcinogenesis, and the relationship between c-Myc and telomerase in human hepatocellular carcinoma tissue is unknown.

**METHODS:** We measured *hTERT* mRNA levels and c-Myc oncoprotein expression in 57 patients with hepatocellular carcinoma using *in situ* hybridization and immunohistochemistry, respectively. The transcription regulation of *hTERT* was evaluated by transient transfection of pGL3-1375 into the human hepatocellular carcinoma cell line J5. To determine the relationship between c-Myc and the *hTERT* promoter, a 1375-bp DNA fragment encompassing the promoter was placed upstream of the luciferase reporter gene and transiently transfected into the cell line. Two additional *hTERT* promoter constructs (-776 and -100 bp region) and an *hTERT* promoter-LUC construct containing 2 c-Myc mutations (pGL3-181 MycMT) were also used for luciferase assays.

**RESULTS:** In 30 of 57 cases (52%), *hTERT* mRNA expression was associated with c-Myc protein expression. However, 16 of 57 cases (28%) showed strong *hTERT* mRNA detection without c-Myc protein expression, and 11 cases (19%) showed weak *hTERT* mRNA expression and strong c-Myc expression. Although luciferase activity was decreased between upstream 1375 bp and 776 bp, there was no significant difference between upstream 776 bp and 100 bp. Finally, there was no significant decrease in activity after transfection of the *hTERT* promoter-LUC construct.

**CONCLUSION:** The results indicate that c-Myc does not play a major role in gene regulation of the catalytic subunit of telomerase (*hTERT*) in human hepatocellular carcinoma.

Other regulatory elements or epigenetic phenomena should be further investigated to understand *hTERT* gene regulation in human hepatocellular carcinoma.

Chen CJ, Kyo S, Liu YC, Cheng YL, Hsieh CB, Chan DC, Yu JC, Harn HJ. Modulation of human telomerase reverse transcriptase in hepatocellular carcinoma. *World J Gastroenterol* 2004; 10 (5): 638-642

<http://www.wjgnet.com/1007-9327/10/638.asp>

## INTRODUCTION

Telomerase is a ribonucleoprotein enzyme that synthesizes G-rich telomeric repeats using its complementary RNA sequence as a template<sup>[1,2]</sup>. Telomerase is expressed in most human cancers and immortal cell lines but is inactive in normal somatic cell lines or tissue<sup>[3-5]</sup>. Recent reports support the concept that activation of telomerase may be an important and obligate step in the development of most malignant tumors<sup>[6,7]</sup>, including human hepatocellular carcinoma (HCC)<sup>[8]</sup>. The human telomerase catalytic subunit (*hTERT*) has been shown to be a rate-limiting determinant of the enzymatic activity of human telomerase<sup>[9,10]</sup>. Takakura *et al* identified the proximal 181-bp core promoter region essential for transactivation of *hTERT*<sup>[11]</sup>. Their findings suggest that *hTERT* expression is strictly regulated at the transcription machinery, and that the proximal core promoter containing an E-box which binds to Myc/Max, as well as the 3' -region containing the GC-box which binds to Sp1, is required for transactivation of *hTERT*<sup>[12]</sup>. Their findings further indicate that c-Myc and Sp1 cooperatively function as the major determinants of *hTERT* expression, and that the switching functions of Myc/Max and Mad/Max might also play roles in telomerase regulation. Wang *et al* added further support that Myc induce telomerase both in normal human mammary epithelial cells and in normal human diploid fibroblasts by introducing HPV-16, E6 protein into these cells<sup>[13]</sup>. Their findings suggest that the ability of c-Myc to activate telomerase may contribute to its ability to promote tumor formation. Further, telomerase activity in estrogen receptor-positive MCF-7 cells was upregulated by treatment with 17 $\beta$ -estradiol<sup>[14]</sup>. Kyo *et al* reported that estrogen activated c-Myc expression in MCF-7 cells, and that E-boxes in the *hTERT* promoter that bind to c-Myc/max played additional roles in estrogen-induced transactivation of *hTERT*.

By using TRAP assay, we previously measured telomerase activity in surgically resected specimens from 25 cases of hepatocellular and adjacent healthy tissues<sup>[15]</sup>. Telomerase activity was detected in 21 of the 25 HCC specimens from 25 different cases. This telomerase activity was correlated with human telomerase reverse transcriptase (*hTERT*) mRNA isoform expression but was poorly related to c-Myc expression in the hepatoma cell line J5<sup>[16]</sup>. However, the role of c-Myc in *hTERT* expression in HCC remains unresolved.

In this study, we explored the relationship between *hTERT* mRNA regulation and c-Myc expression by RNA *in situ* hybridization and immunohistochemistry stain, respectively. The methods of *in situ* hybridization and immunohistochemistry



are semiquantitative and can determine localization. In addition, to determine the cis-elements essential for transcriptional activation of *hTERT*, luciferase assays were performed with reporter plasmids with serial deletions or mutation of the core promoter using hepatoma cell line J5. The results provide evidence for a role of c-Myc in the regulation of *hTERT* in hepatoma cells.

## MATERIALS AND METHODS

### Cell lines

All culture media including fetal bovine serum were purchased from Gibco Laboratories (Grand Island, NY). L-glutamine and penicillin/streptomycin were obtained from Sigma (St. Louis, MO).

WI38 cells (normal human fibroblasts) were obtained from the American Type Culture Collection and grown in DMEM containing 2 mmol/L L-glutamine, 50 U/mL penicillin, 50 mg streptomycin, and 100 mL/L fetal bovine serum. J5<sup>[16]</sup> was maintained in RPMI 1640 medium containing 3 g/L L-glutamine and penicillin/streptomycin. All cell lines were cultivated in an atmosphere of 50 mL/L CO<sub>2</sub> at 37 °C.

### Preparation of RNA probes

Total RNA was obtained from the HT29 cells (ATCC, Rockville, MD) by addition of TRIZOL reagent (Life Technologies, Rockville, MD) according to the manufacturer's instructions.

Ten micrograms of total RNA were used as a template for cDNA synthesis with Moloney murine leukemia virus (M-MTV) reverse transcriptase and oligo (dT)<sub>12-18</sub> (SUPERScript Preamplification System, Life Technologies). Subsequently, the forward primer 5' -CGG AAG AGT GTC TGG AGC AA-3' and the reverse primer 5' -GGA TGA AGC GGA GTC TGG-3' were designed for amplification of a 145-bp segment of *hTERT* spanning from nucleotide position 1 784 to 1 928 (GenBank accessory No. AF015950). Thirty-five PCR cycles were performed. For each cycle, the sample was denatured at 94 °C for 30 s, annealed at 55 °C for 60 s, and extended at 72 °C for 60 s. A 10 µL sample from 100 µL PCR solution was fractionated by electrophoresis on 20 g/L agarose gel. Subsequently, the PCR product was eluted from the agarose gel and subcloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA), generating the construct-designated pCRII/*hTERT*-145.

The sense and antisense riboprobes were synthesized from *Bam* HI- and *Eco* RV-linearized pCRII/*hTERT*-145 according to the manufacturer's instructions using T7 and SP6 RNA polymerase, respectively, and labeled with digoxigenin-UTP (DIG RNA Labeling Kit, SP6/T7, Roche Molecular Biochemicals, Mannheim, Germany). Moreover, the housekeeping gene *GAPDH* was used to confirm the presence of intact RNA within the slides from each sample used for ISH.

### RNA in situ hybridization

Formalin-fixed, paraffin-embedded tissue sections (4-µm thick) were deparaffinized with two 10 min washes with xylene and a graded series of alcohols for 3 min each. The deparaffinized tissues were then pretreated with 20 µg/mL proteinase K (Sigma) and 40 µg/mL pronase (Roche Molecular Biochemicals) at room temperature for 30 min. The tissues were then fixed with 40 g/L paraformaldehyde (Sigma) in phosphate-buffered saline at room temperature for 10 min and then acetylated with 2.5 mL/L acetic anhydride in 0.1 mmol/L triethanolamine-HCl (pH 8.0) at room temperature for 10 min.

Prehybridization was carried out in hybridization solution containing 500 g/L deionized formamide (Merck, Darmstadt, Germany), 5× SSC (1× SSC=150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.2), 1 g/L N-lauroylsarcosine 2 g/L sodium dodecyl sulfate, 20 mL/L blocking solution (DIG wash and block buffer set, Roche Molecular Biochemicals) and 250 µg/mL sonicated salmon sperm DNA (Invitrogen) at 50 °C for 1 h.

The slides were then incubated in a moist chamber at 50 °C for 16 h with the hybridization solution containing 0.1 to 0.5 µg/mL digoxigenin-labeled RNA probe. The slides were subsequently washed twice with 50% formamide-2× SSC at 50 °C for 30 min, twice with 2× SSC at room temperature for 15 min, and twice with 0.2× SSC at room temperature for 15 min. The slides were then equilibrated with 1× washing solution for 2 min and incubated with 10 mL/L blocking solution (DIG wash and block buffer set, Roche Molecular Biochemicals) for 10 min.

The tissues were incubated with a sheep monoclonal antidigoxigenin antibody (Roche Molecular Biochemicals) diluted 1:100 in 10 mL/L blocking solution at room temperature for 2 h. After washed three times with 1× washing solution (Roche Molecular Biochemicals), the color reaction was carried out by incubation with 1× nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate solution (Roche Molecular Biochemicals) at room temperature overnight. The slides were then counterstained with nuclear fast red for 5 min and then mounted with Crystal Mounting reagent (DAKO, Glostrup, Denmark).

Two independent observers evaluated the signal intensity of *hTERT* expression, which was semiquantitated as strong, moderate, weak, or no staining. Sense and antisense probes were applied to paired serial slides, and the noncoding strand detected by sense probes was used as a negative control.

### Immunohistochemistry

Immunohistochemical staining was performed to determine the expression of c-Myc. The immunostaining procedure was performed using the labeled streptavidin-biotin method (LASB-2 Kit, DAKO). Briefly, the tissue was placed in a boiling citrate buffer (pH 6; ChemMate™, DAKO) twice for 5 min in a microwave oven at 750 W after deparaffinization and rehydration, as previously described. Quenching of the endogenous peroxidase activity by incubation with 30 mL/L hydrogen peroxide for 10 min at room temperature was followed by incubation with mouse monoclonal antibody NCL-cMYC (Clone 9E11, Novocastra Laboratories Ltd., Newcastle-upon-Tyne, UK) diluted 1:200 at room temperature for 2 h. After washed with Tris-buffered saline containing 1 g/L Tween-20, the specimens were sequentially incubated for 10 to 30 min with biotinylated anti-mouse immunoglobulins and peroxidase-labeled streptavidin. Staining was performed after 10 min of incubation with a freshly prepared substrate-chromogen solution containing 3% 3-amino-9-ethylcarbazole and hydrogen peroxide. Finally, the slides were lightly counterstained with hematoxylin, washed with water and then mounted. Two independent observers assessed the sections. Because the extent of c-Myc labeling index was heterogeneous, the scoring system included both the staining intensity and the percentage of stained cells<sup>[17]</sup>. Staining intensity was graded as no staining (0), weak (1), moderate (2), or strong (3). The percentage of tumor cells with c-Myc staining was scored as follows: 1, <5%; 2, 5-20%; 3, 21-50%; 4, >50%. The multiplication values were then grouped into 4 scores as 0 (multiplication values 0, 1), 1 (multiplication values 2, 3), 2 (multiplication values 4, 6), or 3 (multiplication values 8, 9, 12).

### PCR amplification and mutation screening of *hTERT* promoter

One microliter of genomic DNA was obtained as DNA template for use in PCR amplification of the *hTERT* promoter. The forward primer 5' -CCC ACG CGT GCA TTC GTG GTG CCC GGA GC-3' and the reverse primer 5' -CCC AGA TCT ATC GCG GGG GTG GCC GGG GCC AGG-3' were designed on the basis of a published *hTERT* promoter sequence<sup>[11]</sup>. The PCR product was amplified in the presence of 1 µmol primers with Taq DNA polymerase (Takara Shuzo Company, Shiga, Japan) for 35 cycles of 1 min at 95 °C, 1 min at 56 °C, and 1 min at 72 °C. DNA sequencing using the reverse primer was

performed directly from the gel-purified PCR product or individual PCR product subcloned into the pCRII-TOPO vector. The analysis of the DNA sequences was compared with the wild-type sequence.

#### Construction of luciferase reporter gene plasmids

A P-3996 construct containing 3 996 bp of sequences upstream of the ATG (with A being position 1) plus exon 1 (219 bp), intron 1 (104 bp), and 37 bp of exon 2 of *hTERT* was used (a kind gift from Silvia Bacchetti, Department of Pathology and Molecular Medicine, McMaster University, Canada)<sup>[18]</sup>. Various lengths of DNA fragments upstream of the initiating ATG codon were PCR amplified and inserted into luciferase reporter vector pGL3-Basic, a promoter- and enhancerless vector (Promega, Madison, WI) in sense orientation relative to the luciferase coding sequence at *MluI* and *BglIII* sites. The sequences of primers were as follows: pGL3-1375-forward: 5'-CCCACGCGTAGACAATTCACAAACACAGC-3', pGL3-776-forward: 5'-CCCACGCGTGCCAGCAGGAGCGCCTGGCT-3', pGL3-100-forward: 5'-CCCACGCGTCCGCGCGGACCCGCCCCGT-3' and reverse (common): 5'-CCCAGATCTATCGCGGGGGTGGCCGGGGCAGGGCTTC-3' with the PCR condition supported by Satoru Kyo (Department of Obstetrics and Gynecology, Kanazawa University, School of Medicine, Ishikawa, Japan)<sup>[11]</sup>. The PCR product was amplified in the presence of 1  $\mu$ L primers with TaKaRa Taq DNA polymerase (Takara Shuzo Company, Shiga, Japan) for 30 cycles of 30 s at 96 °C, 45 s at 62 °C, and 7 min at 72 °C, and 30 min at 72 °C. The products were confirmed to have correct sequences by nucleotide sequencing, and their quantity and quality were routinely checked by agarose gel

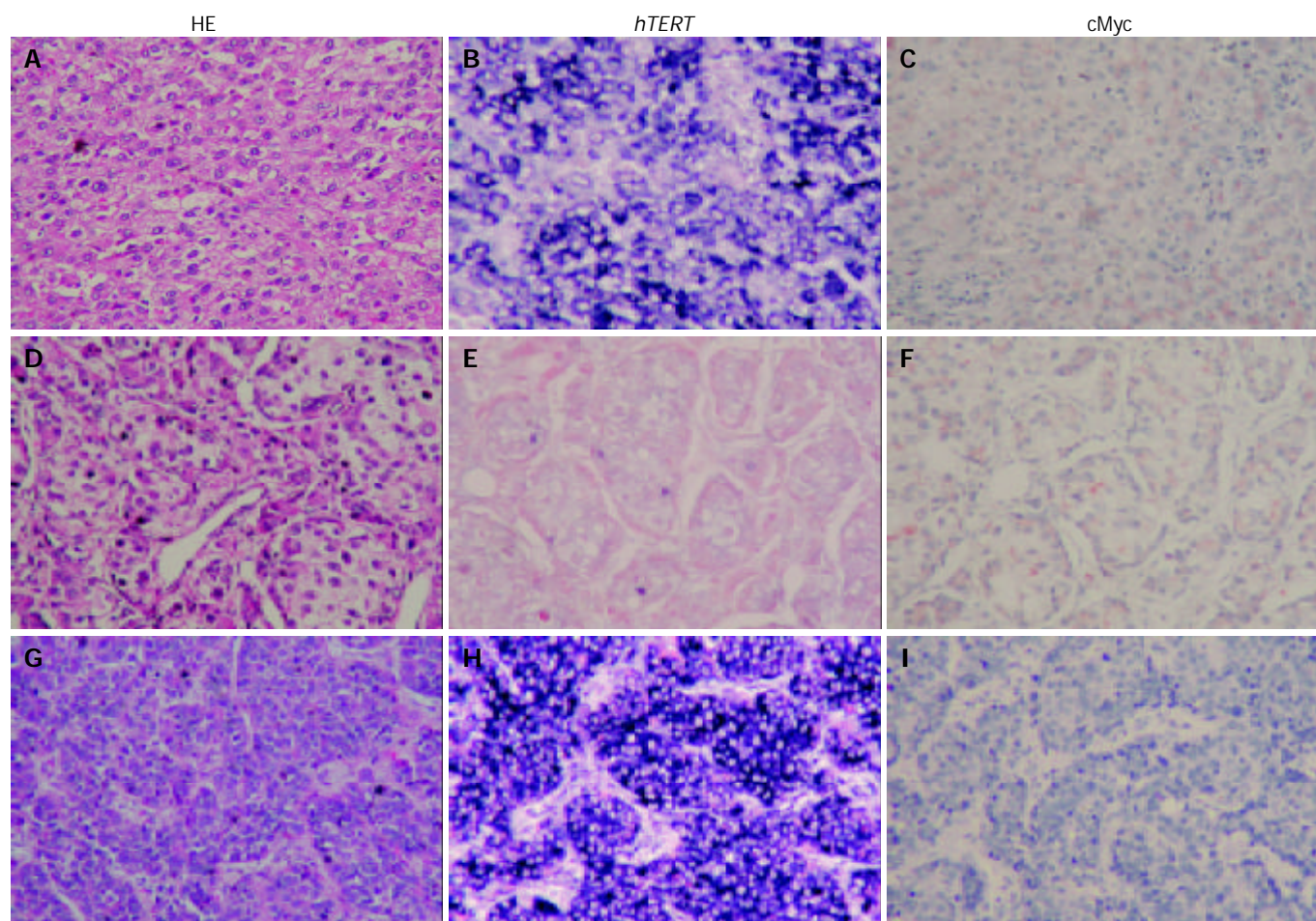
electrophoresis. All plasmid DNAs were purified with the QIAquick gel extraction kit (QIAGEN, Hilden, Germany).

#### Transfection luciferase assay

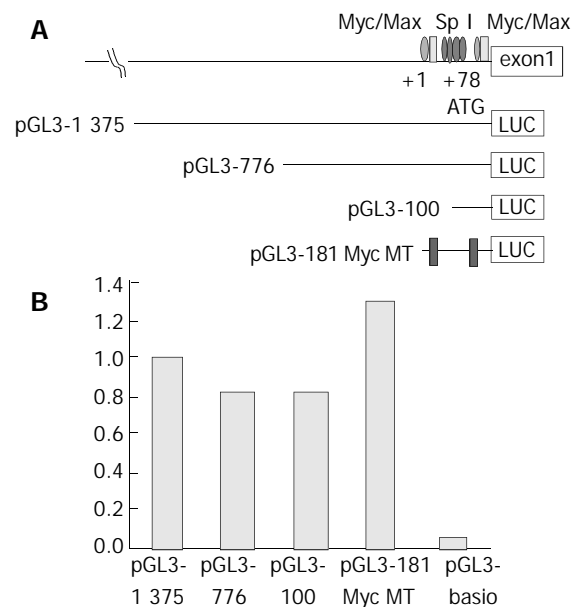
Transient transfection of luciferase reporter plasmids was performed using LipofectAMINE 2000 (LF2000, Invitrogen), according to the protocol recommended by the manufacturer. In brief,  $5 \times 10^4$  cells were seeded on 24-well plates, cultured overnight, and exposed to transfection mixtures containing 2 mg luciferase reporter plasmids for 4 h at 37 °C. Then, 0.5 ml growth media was added and cells were harvested 48 h after transfection. Luciferase assays were performed with the dual-luciferase reporter assay system (Promega) according to the manufacturer's protocols. The pGL3-control plasmid (1 mg/well, Promega) was also transfected into each cell line for better comparison among cell lines with different transfection efficiencies. The pRL-SV40 (1 ng/well, Promega) containing the *Renilla reniformis* luciferase gene was cotransfected with the *hTERT* promoter-luciferase constructs (1 mg/well) for normalization of the luciferase activity in each transfection. The MLX microtiter plate luminometer (Dynex Technologies, Chantilly, VA) was used to detect luciferase activity. All experiments were performed at least 3 times in each plasmid and represented the average relative luciferase activity.

#### Statistical analysis

To evaluate the relationships among paired groups, the Fisher exact test was performed using SPSS 10.0 software. Additionally, the correlation of paired groups was analyzed using chi-square test with the SPSS program. A *P* value  $\leq 0.05$  was considered statistically significant.



**Figure 1** Corresponding distribution of *hTERT* signals and c-Myc staining in HCC tissue detected by HE staining (A, D, and G); *in situ* hybridization (*hTERT* positive, B and H; *hTERT* negative, E); and immunohistochemistry (c-Myc positive, C and F; c-Myc negative, I).



**Figure 2** A: Schematic diagram of LUC reporter plasmids. Promoter fragments of decreasing size from the 5' end (1 375 bp, 776 bp, 100 bp and Myc double deletion mutant *hTERT*-promoter reporter plasmids of 181 bp) upstream of the initiating ATG were inserted into luciferase (*LUC*) reporter vector pGL3-Basic in sense orientation. +1, the transcription start site. Binding sites for c-Myc/Max and Sp1 are shown. B: Transcription activation of *hTERT* promoter. Data represent normalized relative luciferase fold activity compared with the promoterless pGL3 basic plasmid.

## RESULTS

To investigate *hTERT* expression in HCC tissue, *in situ* hybridization was applied. Immunohistochemistry stain was used to observe c-Myc expression and its relationship with *hTERT* mRNA. Forty-seven of 57 cases showed weak to strong *hTERT* mRNA expression. The expression of *hTERT* mRNA was not related to tumor differentiation ( $P < 0.815$ ) (Table 1). Forty-three of 57 cases showed c-Myc expression without tumor differentiation ( $P < 0.348$ ) (Table 2).

**Table 1** Comparison of *hTERT* expression and HCC differentiation

Differentiation	<i>hTERT</i> expression				Number
	None	Weak	Moderate	Strong	
Strong	1	2	2	7	12
Moderate	7	3	4	14	28
Weak	2	4	3	8	17
Number	10	9	9	29	57

**Table 2** Comparison of c-Myc expression and HCC differentiation

Differentiation	c-Myc expression				Number
	0	1	2	3	
Strong	3	0	2	7	12
Moderate	5	7	7	9	28
Weak	6	3	2	6	17
Number	14	10	11	22	57

Thirty of 57 cases (52%) of *hTERT* mRNA expression were associated with c-Myc protein expression (30/57). However, 16 of 57 cases (28%) showed strong *hTERT* mRNA detection

with no Myc protein expression, whereas 11 of 57 cases (19%) showed weak *hTERT* mRNA expression with strong c-Myc detection ( $P < 0.079$ ) (Figure 1).

Three different-length DNA fragments (-1 375, -776, and -100 bp) encompassing the *hTERT* promoter were placed upstream of the luciferase reporter gene, as was an *hTERT* promoter-Luc construct containing 2 c-Myc mutations (pGL-181 MycMT, a gift from Kyo *et al.*). All constructs were transiently transfected into HCC cell line J5 for luciferase study. Luciferase activity decreased between upstream 1 375 and 776 bp, but there was no significant difference of luciferase activity between upstream 776 and 100 bp or the 2 c-Myc mutations (Figure 2).

## DISCUSSION

In a previous report, we demonstrated that telomerase activity in the HCC cell line J5 was not related to c-Myc expression<sup>[16]</sup>. To our knowledge, this is the first study to determine the role of c-Myc in *hTERT* HCC. According to *in situ* hybridization and immunohistochemistry analysis, only half of *hTERT* mRNA expression co-occurred with c-Myc protein expression. Twenty-eight percent of HCC tissue samples had strong *hTERT* mRNA detection with no or weak c-Myc protein expression, 19% of HCC tissue samples had no or weak *hTERT* mRNA expression with strong c-Myc expression. However, several studies reported that Myc expression could transactivate *hTERT* via 2 E-boxes in cooperation with Sp1 motif<sup>[12]</sup>. One of our constructs (a gift from Kyo), which encompassed 4 Sp1 and 2 c-Myc mutations, showed a high luciferase activity in the HCC cell line.

In contrast to the data of Kyo *et al.*<sup>[14]</sup>, pGL3-181MycMT, a double c-Myc mutant, compared with wild type pGL3-181, exhibited a 50% decreased luciferase activity when transfected to the MCF-7 breast cell line. These results implicate that c-Myc is a positive regulator of *hTERT*, though other yet undetermined regulatory elements of *hTERT* in HCC may exist. For example, hepatitis B virus pre-S2/S gene has been found to be a cis-activator of the *hTERT* promoter<sup>[19]</sup>. By transfection of *HBx* gene into the HepG2 cell line, the activity of telomerase and apoptosis were decreased<sup>[20]</sup>. Further investigation of non-c-Myc regulatory proteins in hepatoma is required in the future.

In our 16 HCC tissue specimens and 1 J5 cell line, *hTERT* promoter cis-element sequencing was performed. There was a polymorphism site (A transversion to T) just 3 bp away from the distal E-box, which might have affected the binding affinity of c-Myc<sup>[21]</sup>. This effect might explain why two E-box mutations still had a high telomerase activity. However, more evidence is required in support of this polymorphism nucleotide in 2 E-box mutation construct.

Furthermore, the presence of a large CpG island with a dense CG-rich content implicates that DNA methylation and chromatin structure may play a role in the regulation of *hTERT* expression. Devereux *et al.* demonstrated that the promoter of one *hTERT*-negative fibroblast cell line, SUSM-1, was methylated at all sites examined<sup>[22]</sup>. Treatment of SUM-1 cells with the demethylating agent induced the cells to express *hTERT*, suggesting a potential role for DNA methylation in negative regulation. This epigenetic mechanism could explain why 19% of HCC samples showed strong c-Myc detection with no or weak *hTERT* mRNA expression. The role of GC island methylation in the regulation of *hTERT* expression merits further study.

In the Kyo *et al.* report, the cis-acting effect of E-boxes and the Myc or Max requirement for transactivation varied among different cell types<sup>[12]</sup>. Deletion and mutation of the E-box resulted in significant loss of transcriptional activity in C33A cells, but not in SiHa cells. In C33A cells, expression of

Myc and Max had only marginal effects on transactivation. This diversity among different cell lineages suggests a varied role of c-Myc in *hTERT* gene regulation. The data also indicate that there are multiple levels of regulation of *hTERT* activity in human neoplasm.

In summary, in the present hepatoma tissue study, 50% of hepatomas showed c-Myc overexpression with *hTERT* transcript upregulation. Other regulator elements and epigenetic mechanisms may be involved in *hTERT* transcript regulation. The proximal c-Myc motif plays a minor role in *hTERT* gene regulation. The results of immunohistochemistry and promoter-constructed luciferase analyses suggest that, in HCC, *hTERT* regulation is not restricted to c-Myc and involves other mechanisms.

## REFERENCES

- 1 **Moyzis RK**, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MD, Meyne J, Ratliff RL, Wu JR. A highly conserved repetitive DNA sequence, (TTAGGG)<sub>n</sub>, present at the telomeres of human chromosomes. *Proc Natl Acad Sci U S A* 1988; **85**: 6622-6626
- 2 **Shippen-Lentz D**, Blackburn EH. Functional evidence for an RNA template in telomerase. *Science* 1990; **247**: 546-552
- 3 **Counter CM**, Hirte HW, Bacchetti S, Harley CB. Telomerase activity in human ovarian carcinoma. *Proc Natl Acad Sci U S A* 1994; **91**: 2900-2904
- 4 **Kim NW**, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994; **266**: 2011-2015
- 5 **Shay JW**, Bacchetti S. A survey of telomerase activity in human cancer. *Eur J Cancer* 1997; **33**: 787-791
- 6 **Chadeneau C**, Hay K, Hirte HW, Gallinger S, Bacchetti S. Telomerase activity associated with acquisition of malignancy in human colorectal cancer. *Cancer Res* 1995; **55**: 2533-2536
- 7 **Zhan WH**, Ma JP, Peng JS, Gao JS, Cai SR, Wang JP, Zheng ZQ, Wang L. Telomerase activity in gastric cancer and its clinical implications. *World J Gastroenterol* 1999; **5**: 316-319
- 8 **Tahara H**, Nakanishi T, Kitamoto M, Nakashio R, Shay JW, Tahara E, Kajiyama G, Ide T. Telomerase activity in human liver tissues: comparison between chronic liver disease and hepatocellular carcinomas. *Cancer Res* 1995; **55**: 2734-2736
- 9 **Counter CM**, Meyerson M, Eaton EN, Ellisen LW, Caddle SD, Haber DA, Weinberg RA. Telomerase activity is restored in human cells by ectopic expression of hTERT (hEST2), the catalytic subunit of telomerase. *Oncogene* 1998; **16**: 1217-1222
- 10 **Kim HR**, Christensen R, Park NH, Sapp P, Kang MK, Park NH. Elevated expression of hTERT is associated with dysplastic cell transformation during human oral carcinogenesis *in situ*. *Clin Cancer Res* 2001; **7**: 3079-3086
- 11 **Takakura M**, Kyo S, Kanaya T, Hirano H, Takeda J, Yutsudo M, Inoue M. Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. *Cancer Res* 1999; **59**: 551-557
- 12 **Kyo S**, Takakura M, Taira T, Kanaya T, Itoh H, Yutsudo M, Ariga H, Inoue M. Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT). *Nucleic Acids Res* 2000; **28**: 669-677
- 13 **Wang J**, Xie LY, Allan S, Beach D, Hannon GJ. Myc activates telomerase. *Genes Dev* 1998; **12**: 1769-1774
- 14 **Kyo S**, Takakura M, Kanaya T, Zhuo W, Fujimoto K, Nishio Y, Orimo A, Inoue M. Estrogen activates telomerase. *Cancer Res* 1999; **59**: 5917-5921
- 15 **Hsieh HF**, Harn HJ, Chiu SC, Liu YC, Lui WY, Ho LI. Telomerase activity correlates with cell cycle regulators in human hepatocellular carcinoma. *Liver* 2000; **20**: 143-151
- 16 **Chen CJ**, Tsai NM, Liu YC, Ho LI, Hsieh HF, Yen CY, Harn HJ. Telomerase activity in human hepatocellular carcinoma: parallel correlation with human telomerase reverse transcriptase (hTERT) mRNA isoform expression but not with cell cycle modulators or c-Myc expression. *Eur J Surg Oncol* 2002; **28**: 225-234
- 17 **Brabletz T**, Herrmann K, Jung A, Faller G, Kirchner T. Expression of nuclear beta-catenin and c-myc is correlated with tumor size but not with proliferative activity of colorectal adenomas. *Am J Pathol* 2000; **156**: 865-870
- 18 **Cong YS**, Wen J, Bacchetti S. The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter. *Hum Mol Genet* 1999; **8**: 137-142
- 19 **Horikawa I**, Barrett JC. Cis-Activation of the human telomerase gene (hTERT) by the hepatitis B virus genome. *J Natl Cancer Inst* 2001; **93**: 1171-1173
- 20 **Zhou W**, Shen Q, Gu B, Ren H, Zhang D. Effects of hepatitis B virus X gene on apoptosis and the activity of telomerase in HepG (2) cells. *Zhonghua Ganzangbing Zazhi* 2000; **8**: 212-214
- 21 **O'Hagan RC**, Schreiber-Agus N, Chen K, David G, Engelman JA, Schwab R, Alland L, Thomson C, Ronning DR, Sacchettini JC, Meltzer P, DePinho RA. Gene-target recognition among members of the myc superfamily and implications for oncogenesis. *Nat Genet* 2000; **24**: 113-119
- 22 **Devereux TR**, Horikawa I, Anna CH, Annab LA, Afshari CA, Barrett JC. DNA methylation analysis of the promoter region of the human telomerase reverse transcriptase (hTERT) gene. *Cancer Res* 1999; **59**: 6087-6090

Edited by Wang XL Proofread by Zhu LH



# Prognostic significance of preoperative circulating vascular endothelial growth factor messenger RNA expression in resectable hepatocellular carcinoma: A prospective study

Kuo-Shyang Jeng, I-Shyan Sheen, Yi-Ching Wang, Shu-Ling Gu, Chien-Ming Chu, Shou-Chuan Shih, Po-Chuan Wang, Wen-Hsing Chang, Horng-Yuan Wang

**Kuo-Shyang Jeng**, Department of Surgery, Mackay Memorial Hospital, Taipei, Taiwan, China

**I-Shyan Sheen**, Liver Research Unit, Chang Gung Memorial Hospital, Taipei, Taiwan, China

**Yi-Ching Wang, Shu-Ling Gu, Chien-Ming Chu**, Medical Research, Mackay Memorial Hospital, Taipei, Taiwan, China

**Kuo-Shyang Jeng**, Mackay Junior School of Nursing, Taipei, Taiwan, China

**Shou-Chuan Shih, Po-Chuan Wang, Wen-Hsing Chang, Horng-Yuan Wang**, Department of Internal Medicine, Mackay Memorial Hospital, Taipei, Taiwan, China

**Correspondence to:** I-Shyan Sheen, M.D., Liver Research Unit, Chang Gung Memorial Hospital, No. 199, Tung-Hwa North Road, Taipei, Taiwan, China. issheen.jks@msa.hinet.net

**Telephone:** +886-3-3281200 Ext 8102 **Fax:** +886-2-27065704

**Received:** 2003-10-15 **Accepted:** 2003-12-15

## Abstract

**AIM:** To investigate the prognostic value of vascular endothelial growth factor messenger RNA (VEGF mRNA) in the peripheral blood (PB) of patients with hepatocellular carcinoma (HCC) undergoing curative resection.

**METHODS:** Using a reverse-transcription polymerase chain reaction (RT-PCR)-based assay, VEGF mRNA in the PB was determined prospectively in 50 controls and in 50 consecutive patients undergoing curative resection for HCC.

**RESULTS:** Among the isoforms of VEGF mRNA, VEGF<sub>165</sub> and VEGF<sub>121</sub> were expressed. By multivariate analysis, a higher level of VEGF<sub>165</sub> in preoperative PB correlated with a risk of HCC recurrence with borderline significance ( $P=0.050$ ) and significantly with recurrence-related mortality ( $P=0.048$ ); while VEGF<sub>121</sub> did not. Other significant predictors of HCC recurrence included cellular dedifferentiation ( $P=0.033$ ), an absent or incomplete capsule ( $P=0.020$ ), vascular permeation ( $P=0.018$ ), and daughter nodules ( $P=0.006$ ). The other significant parameter of recurrence related mortality was cellular dedifferentiation ( $P=0.053$ ). The level of circulating VEGF mRNA, however, did not significantly correlate with tumor size, cellular differentiation, capsule, daughter nodules, vascular permeation, necrosis and hemorrhage of tumors.

**CONCLUSION:** The preoperative level of circulating VEGF mRNA, especially isoform VEGF<sub>165</sub>, plays a significant role in the prediction of postoperative recurrence of HCC.

Jeng KS, Sheen IS, Wang YC, Gu SL, Chu CM, Shih SC, Wang PC, Chang WH, Wang HY. Prognostic significance of preoperative circulating vascular endothelial growth factor messenger RNA expression in resectable hepatocellular carcinoma: A prospective study. *World J Gastroenterol* 2004; 10(5): 643-648

<http://www.wjgnet.com/1007-9327/10/643.asp>

## INTRODUCTION

Angiogenesis, known to be essential for the survival, growth, invasion, and metastasis of tumor cells, is a complex multistep process. There are extracellular matrix remodeling and binding of angiogenic factors to specific endothelial cell (EC) receptors, leading to EC proliferation, invasion of the basement membrane, migration, differentiation, and formation of new capillary tubes. Anastomosis of these new vessels develops into a vascular network<sup>[1,2]</sup>.

Several factors with angiogenic activity have been identified, but one of the most potent, direct acting and specific is vascular endothelial growth factor (VEGF), also known as vascular permeability factor and vasculotropin<sup>[3]</sup>.

Hepatocellular carcinoma (HCC), a leading cause of death in Taiwan and many Asian countries, is a highly vascular tumor dependent on neovascularization. Some authors have reported markedly elevated VEGF protein levels in HCC patients with remote metastases compared with those without metastasis, suggesting that VEGF may be a marker for metastasis in HCC<sup>[4-6]</sup>. Most such studies, however, depended on enzyme immunoassay to determine VEGF protein concentrations. To our knowledge, little is known about the prognostic significance of VEGF mRNA expression in the prediction of postresection recurrence of HCC. We conducted this prospective study to investigate the correlation between preoperative VEGF mRNA expression in peripheral blood (PB) and postoperative recurrence of HCC.

## MATERIALS AND METHODS

### Study population

From July 2001 to April 2003, 50 patients (31 men and 19 women, with a mean age of  $56.2 \pm 13.3$  yr) of 58 consecutive patients with HCC undergoing curative hepatectomy were enrolled in this prospective study. Patients who had previously had a hepatectomy or preoperative neoadjuvant ethanol injection or hepatic arterial chemoembolization (TACE) were excluded. The surgical procedures performed included 38 major resections (8 extended right lobectomies, 10 right lobectomies, 8 left lobectomies and 12 two-segmentectomies) and 12 minor resections (10 segmentectomies, 1 subsegmentectomy, and 1 wedge resection). A control group including 30 healthy volunteers without liver disease (15 men, 15 women, mean age 40 yr) and 20 patients with chronic liver disease but without evidence of HCC also gave PB samples.

PB samples for the detection of VEGF mRNA were obtained by forearm venipuncture one day prior to surgery from all 50 patients. After discharge, the patients were assessed regularly to detect tumor recurrence with abdominal ultrasonography (every 2-3 mo during the first 5 yr, then every 4-6 mo thereafter), serum alpha fetoprotein (AFP) and liver biochemistry (every 2 mo during the first 2 yr, then every 4 mo during the following 3 yr, and every 6 mo thereafter), abdominal computed tomography (CT) (every 6 mo during the first 5 yr, then annually), and chest X-ray and bone scans

(every 6 mo). Hepatic arteriography was obtained if the other studies suggested possible cancer recurrence. Detection of tumor on any imaging study was defined as recurrence.

Clinicopathological parameters analyzed included sex (male vs female), age, the presence of liver cirrhosis, hepatitis B virus (HBV) infection (hepatitis B surface antigen), hepatitis C virus (HCV) infection (anti-hepatitis C virus antibody), serum AFP level (<20 ng/mL vs 20 to 1 000 ng/mL vs >1 000 ng/mL), cirrhosis, Child-Pugh class of liver functional reserve (A vs B), tumor size (<3 cm vs 3 to 10 cm vs >10 cm), tumor encapsulation (complete vs incomplete or absent), presence of daughter nodules, vascular permeation (including vascular invasion and/or tumor thrombi in either the portal or hepatic vein), and cell differentiation grade (Edmondson and Steiner grades I to IV).

### Detection of VEGF mRNA

Ethylenediamine tetraacetic acid (EDTA)-treated whole blood was centrifuged and the plasma fraction removed. The cellular fraction was enriched for mononuclear cells or possible tumor cells according to the method described by Oppenheim. Nucleated cells were isolated from peripheral blood using tetradecyltrimethyl-ammonium bromide. Total cellular RNA was then extracted with PUREscript RNA Isolation Kits TRI-Zol (Life Technologies Inc., Gaithersburg, USA). cDNA was synthesized from 5 µg of the mRNA. The reverse transcription reaction solution contained 6 µL of 5×first strand buffer, 10 mmol/L dithiothreitol, 125 mmol/L each of dCTP, dATP, dGTP and dTTP, 0.3 µg of random hexamers, and 200 units of Superscriptase II Moloney murine leukemia virus reverse transcriptase (Life Technologies Inc.). The RNA solution was incubated at 95 °C for 10 min, quickly chilled on ice, then mixed with the reverse transcription reaction solution (total volume 20 µL), and incubated at 37 °C for 60 min. The sequences of the sense primers were 5'-AGTGTGTGCCCCACTGAGGA-3' (VEGF) and 5'-AGTCAACGGATTTGGTCGTA-3' (GAPDH) and those of the antisense primers were 5'-AGTCAACGGATTTGGTCGTA-3' (VEGF) and 5'-GGAACATGTAAACCATGTAG-3' (GAPDH). The first polymerase chain reaction (RT-PCR) solution contained 5 µL of the synthesized cDNA solution, 10 µL of 10× polymerase reaction buffer, 500 mol/L each of dCTP, dATP, dGTP and dTTP, 15 pmol of each external primer (EX-sense and EX-antisense), 4 units of Thermus Brockiamus Prozyme DNA polymerase (PROtech Technology Ent. Co., Ltd. Taipei, Taiwan), and water. The PCR cycles were: denaturing at 94 °C for 1 min, annealing at 52 °C for 1 min, and primer extension at 72 °C for 1 min. The cycles were repeated 40 times. The PCR product was reamplified with internal primers for nested PCR to obtain a higher sensitivity. The first and second PCR components were the same, but for the primer pairs (IN-sense and IN-antisense), the final product was electrophoresed on 20g/L agarose gel and stained with ethidium bromide. Four different isoforms of human VEGF were identified, arising from alternative splicing of the primary transcript of a single gene. The majority were VEGF<sub>121</sub> (165 bp) and VEGF<sub>165</sub> (297 bp). The percentage intensity of the VEGF PCR fragment for each liver was relative to a GAPDH PCR fragment (122 bp). The intensity of bands was measured using Fujifilm Science Lab 98 (Image Gauge V3.12). The sensitivity of our assay was assessed using human hepatocytes.

A HepG2 (hepatoblastoma) cell line served as a positive control for VEGF mRNA expression. For negative controls, we used EDTA-treated water (filtered and vaporized).

### Statistical analysis

A statistical software (SPSS for Windows, version 8.0, Chicago, Illinois) was employed, with Student's *t*-test used to analyze

continuous variables and a chi-square or Fisher's exact test for categorical variables. Factors relating to the presence of postoperative hAFP mRNA in peripheral blood were analyzed by stepwise logistic regression. A Cox proportional hazards model was used for multivariate stepwise analysis to identify significant factors for predicting recurrence and mortality. Significance was taken as a *P* value <0.05.

## RESULTS

### RT-PCR analysis of VEGF transcript in peripheral blood

VEGF mRNA was expressed in the peripheral blood of 10 (VEGF<sub>165</sub> in 4 and VEGF<sub>121</sub> in 10) of 50 control patients (10/50, 20%). In the HCC group, isoform VEGF<sub>165</sub> was detected in 40 patients (80%) (with a concentration ranging from 0.198 to 0.7190) and isoform VEGF<sub>121</sub> in all 50 patients (100%) (concentration ranging from 0.2958 to 1.0356).

We did not detect isoforms VEGF<sub>189</sub> and/or VEGF<sub>206</sub> in either study or control patients

**Table 1** Demographic, clinical and tumor variables of patients with HCC undergoing curative resection (*n*=50)

Variables	No. of patients (%)
Age (mean, years)	56.2±13
Male	31 (62)
Child- Pugh's class A	43 (86)
Serum AFP <20 ng/mL	16 (32)
20-10 <sup>3</sup> ng/mL	18 (36)
>10 <sup>3</sup> ng/mL	14 (28)
HBsAg (+)	36 (72)
Anti-HCV (+)	13 (26)
Size of HCC <3 cm	12 (24)
3-10 cm	13(26)
>10 cm	25(50)
Cirrhosis	40(80)
Edmondson-Steiner's Grade I	4 (8)
Grade II	12 (24)
Grade III	18 (36)
Grade IV	16 (32)
Complete capsule	19 (38)
Vascular permeation	29 (58)
Daughter nodules	31 (62)
Tumor necrosis	33(66)
Tumor hemorrhage	29(58)

AFP: serum alpha fetoprotein; HBsAg (+): positive hepatitis B surface antigen; Anti-HCV(+): positive hepatitis C virus antibody; Edmondson Steiner grade: differentiation grade.

### Correlation of VEGF mRNA expression and tumor recurrence

Sixteen patients (32%) had clinically detectable recurrence during the follow up period (median 1.5 yr, range 1 to 2.5 yr), of whom 7 died. A high preoperative level of isoform VEGF<sub>165</sub> mRNA correlated significantly with tumor recurrence both univariately (*P*=0.021) and multivariately (*P*=0.050). Isoform VEGF<sub>121</sub> levels had no such correlation. By multivariate analysis, other significant predictors of recurrence included poor cellular differentiation (*P*=0.033), less encapsulation (*P*=0.020), more vascular permeation (*P*=0.018) and the presence of daughter nodules (*P*=0.006) (Table 2).

### Correlation of VEGF mRNA expression and recurrence-related death

The preoperative level of isoform VEGF<sub>165</sub> in PB significantly correlated with death from recurrence both univariately

( $P<0.001$ ) and multivariately ( $P=0.048$ ). By multivariate analysis, a greater degree of vascular permeation significantly correlated with mortality ( $P=0.045$ ), and poor cellular differentiation approached significance ( $P=0.053$ ) (Table 3).

**Table 2** Predictors of HCC recurrence

Variable	P values	
	UV	MV
Sex	0.895	-
Age	0.279	-
Size(<3 cm,>10 cm)	0.415	-
Liver cirrhosis	0.510	-
Child-Pugh class	0.528	-
Serum AFP	0.744	-
HBsAg (+)	0.280	-
Anti-HCV (+)	0.481	-
Edmondson Steiner grade	0.0005	0.033
Capsule	<0.0001	0.020
Vascular permeation	<0.0001	0.018
Daughter nodules	<0.0001	0.006
Tumor necrosis	0.344	-
Tumor hemorrhage	0.812	-
Serum VEGF <sub>165</sub> mRNA	0.0206	0.050
Serum VEGF <sub>121</sub> mRNA	0.520	-

UV: univariate analysis; MV: multivariate analysis; AFP: serum alpha fetoprotein; HBsAg(+): positive hepatitis B surface antigen; Anti-HCV(+): positive hepatitis C virus antibody; Edmondson Steiner grade: differentiation grade I, II vs III, IV; n.s: not significant.

**Table 3** Correlation between clinical and tumor variables and recurrence-related mortality

Variables	P values	
	UV	MV
Sex	0.510	-
Age	0.440	-
Size (<3 cm, >10 cm)	0.519	-
Liver cirrhosis	0.510	-
Child-Pugh class	0.548	-
HBsAg (+)	0.351	-
Anti-HCV (+)	0.521	-
Edmondson Steiner grade	<0.001	0.053
Capsule	0.033	n.s.
Vascular permeation	<0.001	0.045
Daughter nodules	0.016	n.s.
Tumor necrosis	0.373	-
Tumor hemorrhage	0.306	-
Serum VEGF <sub>165</sub> mRNA	<0.001	0.048
Serum VEGF <sub>121</sub> mRNA	0.763	-

UV: univariate analysis; MV: multivariate analysis; AFP: serum alpha fetoprotein; HBsAg (+): positive hepatitis B surface antigen; Anti-HCV(+): positive hepatitis C virus antibody; Edmondson Steiner grade: differentiation grade I, II vs III, IV; n.s: not significant.

#### Correlation between VEGF mRNA expression and clinical and histopathologic features

There was no significant association between either isoform of VEGF mRNA and gender, age, serum AFP level, chronic HBV or HCV carriage, tumor size, coexisting cirrhosis, cellular differentiation, capsule, vascular permeation, daughter nodules, tumor necrosis, or tumor hemorrhage (all  $P>0.05$ ).

## DISCUSSION

Our study showed that a higher value of circulating VEGF mRNA isoform <sub>165</sub> before curative resection of HCC was significantly associated with an increased risk of postoperative recurrence and disease mortality. The presence of preoperative VEGF mRNA isoform <sub>121</sub> was not significantly predictive of outcomes.

The active form of VEGF was a homodimeric cytokine with molecular weight of 34-46 ku, the variation in size was due to alternative exon splicing which produces four different isoforms of 121, 165, 189 and 206 amino acids (monomeric size). The last three of those had heparin binding activity<sup>[15]</sup>. Different cancers have different expression of the isoforms. The majority of HCC could express an abundance of VEGF <sub>121</sub> and VEGF <sub>165</sub><sup>[4,7,8]</sup>. Further analysis by Ferrara indicated that VEGF<sub>165</sub> was the predominantly expressed form in human cDNA libraries as well as in most normal cells and tissues<sup>[8]</sup>.

Several studies have revealed that the serum VEGF level is of value for predicting disease progression and prognosis in cancers of different origins, including the breast, gastrointestinal organs, kidney, urothelium, ovary, lung, and lymphoma<sup>[9-18]</sup>. However, the serum level of VEGF, which is what most other investigators have measured, might be influenced by other factors such as platelet number, associated liver cirrhosis, or coexisting infection<sup>[19-22]</sup>. For accurate measurement, citrated plasma processed within 1 h of venipuncture is better than serum.

Measuring VEGF in HCC tissue has the disadvantage of not being available until after a biopsy or resection has been done. The majority of angiogenic factors are soluble, diffusible peptides. Hence, the circulating level of angiogenic factors theoretically reflects the angiogenic activity of the tumor. Compared with expression in tumor tissue, the advantage of the measurement of circulating VEGF expression is that it can be performed without tissue specimens and repeated serially. Jinno *et al.*, believed that circulating VEGF might be derived mainly from a large burden of tumor cells, but also partly from platelets activated by vascular invasion of HCC cells<sup>[20]</sup>.

We chose to measure circulating mRNA expression of VEGF rather than the protein itself. According to El-Assal's study, the level of VEGF mRNA did not always correlate with the protein concentration<sup>[23]</sup>. Immunohistochemistry could not distinguish small amounts of protein, which may partly explain the discrepancy in protein and mRNA levels.

An additional question is whether the value of circulating VEGF (either protein or mRNA) corresponds with VEGF mRNA in HCC tissue. Tokunaga noted that the circulating VEGF mRNA isoform pattern in colon cancer was not always significantly correlated with the gene expression level. The release of VEGF mRNA might be influenced by some cells other than HCC cells<sup>[24]</sup>. Warren found VEGF mRNA in hepatocytes and some Kupffer cells<sup>[25]</sup>. According to Banks, the presence of mRNA for VEGF was also described in T lymphocytes, CD34\* cells, and monocytes<sup>[19,21]</sup>.

There are considerable discrepancies among reports about the clinical significance of VEGF expression in HCC<sup>[20,26-34]</sup>. The high recurrence rate after resection has been found to be the main determinant for the poor outcome of HCC<sup>[35-37]</sup>. Tumor invasiveness variables correlating with recurrence include high serum AFP, hepatitis, vascular permeation, the grade of cellular differentiation, infiltration or absence of capsule, tumor size, coexisting cirrhosis, the presence of daughter nodules, and multiple lesions. Therefore, a number of studies have been done to see if VEGF correlated with any or all of those factors.

Zhou showed that high VEGF expression in HCC was associated with portal vein tumor thrombosis<sup>[28]</sup>. Li reported that VEGF mRNA in HCC correlated significantly with portal



vein emboli, poorly encapsulated tumors, and microvascular density in HCC tissues<sup>[27]</sup>. Chow found that VEGF expression was significantly associated with the PCNA index and sonographic evidence of portal vein tumor thrombosis but not with the liver biochemical profile, tumor volume, gender, severity of liver disease, or tumor grading<sup>[26]</sup>.

One possible explanation for the discrepancies may be the assessment of tumors of different sizes and etiologies. The number of study patients is another possible factor. Because most of the reported investigations were performed in small series, we used 50 patients which seemed an adequate sample size compared with other studies. It is also likely that the effect of VEGF on angiogenesis depends on not only tumor cell expression of VEGF, but also on the VEGF receptors in endothelial cells.

Some have reported a correlation between increased plasma VEGF protein level in HCC patients and tumor size, number, portal vein emboli, poorly encapsulated tumors, microscopic venous invasion, metastasis, and recurrence. Yamamoto found there was a positive correlation between the increment of intratumoral MVD and serum VEGF concentrations<sup>[38]</sup>.

According to our study, a higher expression of circulating VEGF mRNA was significantly correlated with tumor recurrence and recurrence-related mortality but not with the other accepted measures of invasiveness. Circulating VEGF mRNA thus appears to be an independent risk factor of postoperative recurrence. There are several possible explanations for this dissociation.

Some studies have found that the histologic grade of HCC was associated with VEGF expression in noncancerous liver cells, suggesting a complex regulatory mechanism for circulating VEGF in liver disease. Coexisting liver cirrhosis might influence VEGF expression<sup>[22,23]</sup>. About 80% of our study patients had cirrhosis. Some investigators have found that VEGF expression was significantly higher in cirrhotic livers than in noncirrhotic livers. Furthermore, it has been shown that cirrhosis itself was associated with increased angiogenic activity. El-Assal *et al* observed that cirrhotic livers had significantly higher VEGF expressions than noncirrhotic livers, suggesting that VEGF might be associated with angiogenesis in cirrhosis<sup>[23]</sup>. In addition, some suggested a possible involvement of VEGF in angiogenesis of the cirrhotic liver but not in angiogenesis of HCC. Akiyoshi suggested that a low serum VEGF level in liver cirrhosis might reflect the degree of liver dysfunction and might be associated with the grade of hepatocyte regeneration<sup>[22]</sup>. VEGF levels were decreased with the worsening of the Child-Pugh score. However, most of our patients were Child-Pugh class A, and their resectable lesions, were unlike those studied by Akiyoshi.

The stage of cancer might influence the VEGF expression<sup>[38-41]</sup>. Chao showed that a lower range of circulating VEGF levels in patients with early-stage HCC overlapped considerably with those in normal controls or in patients with chronic hepatitis or cirrhosis<sup>[30]</sup>. Therefore, serum VEGF is probably not useful for early detection of HCC. In contrast, a large quantity of VEGF may be released by the large load of HCC cells in advanced disease (stage VI B). Because plasma VEGF level is significantly higher in stage IVB than in stage IVA, other mechanisms may be responsible, such as agglutination and activation of platelets caused by vascular invasion and circulating tumor cells. It may thus be the platelets rather than or in addition to HCC cells that are responsible for the release of VEGF into circulation. Our patients did not have such an advanced disease.

The relation between tumor size and VEGF mRNA expression might be complex and dynamic because of different vascular growth patterns<sup>[5,34,42-44]</sup>. In small hypervascular HCCs, approximately 1.0 cm in diameter in those that grow with a pattern of vessel replacement, artery-like vessels are not well

developed. Capillarization of the blood spaces is present but in an incomplete form, and portal tracts often appear within cancerous nodules. These HCCs are thought to receive a predominantly portal blood supply. As tumor size increases, portal tracts decrease in number, and artery-like vessels gradually increase in number and size. Well-differentiated HCCs measuring 1.0 to 1.5 cm in diameter are in a transitional stage from portal to arterial blood supply, with the reduction in portal flow preceding the increase in arterial flow. Therefore, blood flow in HCC at this point would be low. This may be the reason why many well-differentiated HCCs are not detected on angiography, with hypervascularity seen until nodules become larger than 2 cm in diameter.

VEGF positivity may therefore gradually decrease with increasing tumor size. According to Yamaguchi, 36.8% of nodules larger than 3.0 cm were VEGF-negative<sup>[34]</sup>. El-Assal showed that, contrary to the usual angiographic findings, HCCs larger than 5 cm in diameter were not more vascular than smaller tumors and were less vascular than medium-sized lesions<sup>[23]</sup>. However, it has been reported that the intercapillary distance increased as the tumors size or weight increased, which may be caused by the significantly different rates of endothelial (50 to 60 h) and neoplastic cell (22 h) turnover.

These complicated changes in vascularity may account for the disparate results among reported studies. Suzuki reported that VEGF mRNA levels were not correlated with the vascularity of HCCs as seen on angiography<sup>[4]</sup>. On the contrary, Mise *et al*. showed that the degree of VEGF mRNA expression was significantly correlated with the intensity of tumor staining in angiograms ( $P < 0.01$ )<sup>[5]</sup>. Some have reported higher VEGF expression in small-size and well-differentiated HCCs and suggested that VEGF played its most important role in a relatively early stage of angiogenesis. In general, advanced HCCs are mainly supplied with arterial blood, and arterial angiography may reveal hypervascularity. Serum VEGF concentrations have been reported to be significantly higher in advanced stage rather than in early stage of breast and gastric cancer<sup>[9,13,14]</sup>.

Neovascularization appears to be one of the crucial steps in a tumor's transition from a small, harmless cluster of mutated cells to a large, malignant growth, capable of spreading to other organs throughout the body. Because of the complex nature of the angiogenic process, however, it seems that VEGF expression is not the sole contributor to angiogenesis in HCC. Other factors involved in this process may include TGF- $\beta$ , TNF- $\alpha$ , IL-8, *etc.*

It has been reported recently that VEGF mRNA expression was readily induced by hypoxia or ischemia. This is why we excluded patients who received preoperative TACE, in order to avoid ischemia-induced changes in VEGF expression.

Although tumor necrosis was present in 66% of our study patients (Table 1), circulating VEGF mRNA did not statistically correlate with it. We also did not find any correlation with fibrous capsule or septum formation, in contrast to the findings of Inoue *et al*<sup>[12]</sup>. The origin of the capsule and fibrous septa in HCC is unclear. Nakashima *et al* suggested the possibility of fibrogenesis at the interface of two tumor nodules of different properties, a process requiring fibrin deposition in the initial stage<sup>[45]</sup>. Some authors stated that a capsule or septa was formed when the HCC nodule grew to 1.5 cm or larger. It has been suggested that capsule formation is a result of compression and collagenization of the adjacent stroma. However, this mechanism has been doubted, since the tumor size did not correlate with the thickness of the capsule or the incidence of its formation. This suggests that capsules are formed by active fibrosis rather than by tumor compression on the adjacent stroma.

According to our study, circulating VEGF mRNA did not significantly correlate with the grade of cellular differentiation.

We attributed this to the possibility of different histological grades coexisting in HCC tissues. Yamaguchi examined VEGF expression immunohistochemically in HCC of various histological grades and sizes<sup>[34]</sup>. In tumors composed of a single histological grade, VEGF expression was the highest in well-differentiated, followed by moderately differentiated, and then poorly differentiated HCC. In tumors consisting of cancerous tissues of two different histological grades, the expression was less intense in the higher-grade HCC component. VEGF was also expressed in the surrounding HCC tissues in which inflammatory cell infiltration was apparent. Based on these findings, VEGF expression in HCC tissues was thought to be partly related to the histological grade but other cytokines and growth factors could also cooperatively act to enhance or influence VEGF expressions in HCC.

Solid tumors such as HCC are composed of two distinct compartments, namely the malignant cells themselves and the vascular and connective tissue stroma. The stroma provides the vascular supply that tumors require for obtaining nutrients, gas exchange, and waste disposal. Fibrin serves as a provisional stroma that is gradually replaced by granulation tissue and then by mature stroma. Human HCC is commonly surrounded by a fibrous capsule with an abundant extracellular matrix, even in the early stage. It is possible that VEGF also plays a certain role in the stimulation of regional development of the stroma in HCC.

Angiogenesis also appears to be involved in the invasion of tumors into the surrounding tissues, because this invasion requires concomitant neovascularization through the sprouting of endothelial cells in the extracellular matrix. It has been reported that VEGF induced both urokinase-type and tissue-type plasmin in endothelial cells. These are the key proteases involved in the degradation of the extracellular matrix. Thus VEGF may promote the process of vascular invasion by HCC cells.

Some authors suggested that VEGF mRNA expression in PB, which correlates well with the shift of VEGF mRNA in liver tissue, was strongly related to the development of HCC, including the progression from preneoplastic to neoplastic tissue and the potential of post-resection recurrence, the invasiveness of HCC, and poor survival<sup>[42-44]</sup>. Some stated that serum VEGF was a predictor of invasion and metastasis of HCC and a potential biomarker of metastatic recurrence after curative resection<sup>[20]</sup>.

Surgery remains the best potentially curative treatment for patients with HCC. High recurrence rate limits the long term survival. Examination of preoperative VEGF mRNA in PB expression may give us information about high risk of postoperative recurrence. Addition of neoadjuvant or antiangiogenic therapy before or after surgery may be considered for such patients. Furthermore, the serial measurement of circulating VEGF mRNA during postoperative follow-up to monitor the effect of therapy or the development of recurrence needs further investigation.

From this prospective study, we suggest that circulating VEGF mRNA expression, especially isoform VEGF<sub>165</sub>, may play a significant role in the prediction of postresection recurrence of HCC.

## ACKNOWLEDGEMENT

This study was supported by grants from the Department of Medical Research, Mackay Memorial Hospital, Taiwan (MMH 9237).

## REFERENCES

- Folkman J.** Endothelial cells and angiogenic growth factors in cancer growth and metastasis. *Cancer Metastasis Rev* 1990; **9**: 171-174
- Zetter BR.** Angiogenesis and tumor metastasis. *Annu Rev Med* 1998; **49**: 407-424
- Dvorak HF, Brown LF, Detmar M, Dvorak AM.** Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995; **146**: 1029-1039
- Suzuki K, Hayashi N, Miyamoto Y, Yamamoto M, Ohkawa K, Ito Y, Sasaki Y, Yamaguchi Y, Nakase H, Noda K, Enomoto N, Arai K, Yamada Y, Yoshihara H, Tujimura T, Kawano K, Yoshikawa K, Kamada T.** Expression of vascular permeability factor/vascular endothelial growth factor in human hepatocellular carcinoma. *Cancer Res* 1996; **56**: 3004-3009
- Mise M, Arai S, Higashitani H, Furutani M, Niwano M, Harada T, Ishigami S, Toda Y, Nakayama H, Fukumoto M, Fujita J, Imamura M.** Clinical significance of vascular endothelial growth factor and basic fibroblast growth factor gene expression in liver tumor. *Hepatology* 1996; **23**: 455-464
- Miura H, Miyazaki T, Kuroda M, Oka T, Machinami R, Kodama T, Shibuya M, Makuuchi M, Yazaki Y, Ohnishi S.** Increased expression of vascular endothelial growth factor in human hepatocellular carcinoma. *J Hepatol* 1997; **27**: 854-861
- Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW.** The vascular endothelial growth factor family: identification of a fourth molecular species and characterization molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol* 1991; **5**: 1806-1814
- Ferrara N, Houck K, Jakeman L, Leung DW.** Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocrinol Rev* 1992; **13**: 18-32
- Brown LF, Berse B, Jackman RW, Tognazzi K, Guidi AJ, Dvorak HF, Senger DR, Connolly JL, Schnitt SJ.** Expression of vascular endothelial permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. *Hum Pathol* 1995; **26**: 86-91
- Anan K, Morisaki T, Katano M, Ikubo A, Kitsuki H, Uchiyama A, Kuroki S, Tanaka M, Torisu M.** Vascular endothelial growth factor and platelet-derived growth factor are potential angiogenic and metastatic factors in human breast cancer. *Surgery* 1996; **119**: 333-339
- Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM.** Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995; **55**: 3964-3968
- Inoue K, Ozeki Y, Suganuma T, Sugiura Y, Tanaka S.** Vascular endothelial growth factor expression in primary esophageal squamous cell carcinoma: association with angiogenesis and tumor progression. *Cancer* 1997; **79**: 206-213
- Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Senger DR, Dvorak HF.** Expression of vascular permeability factor (vascular endothelial growth factor) and its receptor in adenocarcinomas of the gastrointestinal tract. *Cancer Res* 1993; **53**: 4727-4735
- Maeda K, Chung YS, Ogawa Y, Takatsuka S, Kang SM, Ogawa M, Sawada T, Sowa M.** Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. *Cancer* 1996; **77**: 858-863
- Salven P, Ruotsalainen T, Mattson K, Joensuu H.** High pre-treatment serum level of vascular endothelial growth factor (VEGF) is associated with poor outcome in small-cell lung cancer. *Int J Cancer* 1998; **79**: 144-146
- Miyake H, Hara I, Yamanaka K, Gohji K, Arakawa S, Kamidono S.** Elevation of serum level of vascular endothelial growth factor as new predictor of recurrence and disease progression in patients with superficial urothelial cancer. *Urology* 1999; **53**: 302-307
- Tempfer C, Obrmair A, Heffler L, Haeusler G, Gitsch G, Kainz C.** Vascular endothelial growth factor serum concentrations in ovarian cancer. *Obstet Gynecol* 1998; **92**: 360-363
- Salven P, Teerenhovi L, Joensuu H.** A high pretreatment serum vascular endothelial growth factor concentration is associated with poor outcome in non-Hodgkin's lymphoma. *Blood* 1997; **90**: 3167-3172
- Banks RE, Forbes MA, Kinsey SE.** Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: bearing human hepatocellular carcinoma. *J Cancer Res Clin Oncol* 1997; **123**: 383-387
- Jin-no K, Tanimizu M, Hyodo I, Nishikawa Y, Hosokawa Y, Doi T, Endo H, Yamashita T, Okada Y.** Circulating vascular endot-

- helial growth factor (VEGF) is a possible tumor marker for metastasis in human hepatocellular carcinoma. *J Gastroenterol* 1998; **33**: 376-382
- 21 **Banks RE**, Forbes MA, Kinsey SE, Stanley A, Ingham E, Walters C, Selby PJ. Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: significance for VEGF measurements and cancer biology. *Br J Cancer* 1998; **77**: 956-964
- 22 **Akiyoshi F**, Sata M, Suzaki H, Uchimura Y, Mitsuyama K, Matsuo K, Tanikawa K. Serum vascular endothelial growth factor levels in various liver diseases. *Dig Dis Sci* 1998; **43**: 41-45
- 23 **El-Assal ON**, Yamanoi A, Soda Y, Yamaguchi M, Igarashi M, Yamamoto A, Nabika T, Nagasue N. Clinical significance of microvessel density and vascular endothelial growth factor expression in hepatocellular carcinoma and surrounding liver: possible involvement of vascular endothelial growth factor in the angiogenesis of cirrhotic liver. *Hepatology* 1998; **27**: 1554-1562
- 24 **Tokunaga T**, Oshika Y, Abe Y, Ozeki Y, Sadehiro S, Kijima H, Tsuchida T, Yamazaki H, Ueyama Y, Tamaoki N, Nakamura M. Vascular endothelial growth factor (VEGF) mRNA isoform expression pattern is correlated with liver metastasis and poor prognosis in colon cancer. *Br J Cancer* 1998; **77**: 998-1002
- 25 **Warren RS**, Yuan H, Matli MR, Gillett NA, Ferrara N. Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest* 1995; **95**: 1789-1797
- 26 **Chow NH**, Hsu PI, Lin XZ, Yang HB, Chan SH, Cheng KS, Huang SM, Su JJ. Expression of vascular endothelial growth factor in normal liver and hepatocellular carcinoma: an immunohistochemical study. *Hum Pathol* 1997; **28**: 698-703
- 27 **Li XM**, Tang ZY, Zhou G, Lui YK, Ye SL. Significance of vascular endothelial growth factor mRNA expression in invasion and metastasis of hepatocellular carcinoma. *J Exp Clin Cancer Res* 1998; **17**: 13-17
- 28 **Zhou J**, Tang ZY, Fan J, Wu ZQ, Li XM, Liu YK, Liu F, Sun HC, Ye SL. Expression of platelet-derived endothelial cell growth factor and vascular endothelial growth factor in hepatocellular carcinoma and portal vein tumor thrombus. *J Cancer Res Clin Oncol* 2000; **126**: 57-61
- 29 **Qin LX**, Tang ZY. The prognostic molecular markers in hepatocellular carcinoma. *World J Gastroenterol* 2002; **8**: 385-392
- 30 **Chao Y**, Li CP, Chau GY, Chen CP, King KL, Lui WY, Yen SH, Chang FY, Chan WK, Lee SD. Prognostic significance of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin in patients with resectable hepatocellular carcinoma after surgery. *Ann Surg Oncol* 2003; **10**: 355-362
- 31 **Miura H**, Miyazaki T, Kuroda M, Oka T, Machinami R, Kodama T, Shibuya M, Makuuchi M, Yazaki Y, Ohnishi S. Increased expression of vascular endothelial growth factor in human hepatocellular carcinoma. *J Hepatol* 1997; **27**: 854-861
- 32 **Torimura T**, Sata M, Ueno T, Kin M, Tsuji R, Suzaku K, Hashimoto O, Sugawara H, Tanikawa K. Increased expression of vascular endothelial growth factor is associated with tumor progression in hepatocellular carcinoma. *Hum Pathol* 1998; **29**: 986-991
- 33 **Motoo Y**, Sawabu N, Nakanuma Y. Expression of epidermal growth factor and fibroblast growth factor in human hepatocellular carcinoma: an immunohistochemical study. *Liver* 1991; **11**: 272-277
- 34 **Yamaguchi R**, Yano H, Iemura A, Ogasawara S, Haramaki M, Kojiro M. Expression of vascular endothelial growth factor in human hepatocellular carcinoma. *Hepatology* 1998; **28**: 68-77
- 35 **Poon RT**, Fan ST, Lo CM, Liu CL, Wong J. Intrahepatic recurrence after curative resection of hepatocellular carcinoma. Long-term results of treatment and prognostic factors. *Ann Surg* 1999; **229**: 216-222
- 36 **Jeng KS**, Sheen IS, Chen BF, Wu JY. Is the p53 gene mutation of prognostic value in hepatocellular carcinoma after resection? *Arch Surg* 2000; **135**: 1329-1333
- 37 **Ng IO**, Lai EC, Fan ST, Ng MM, So MK. Prognostic significance of pathologic features of hepatocellular carcinoma. *Cancer* 1995; **76**: 2443-2448
- 38 **Yamamoto Y**, Toi M, Kondo S, Matsumoto T, Suzuki H, Kitamura M, Tsuruta K, Taniguchi T, Okamoto A, Mori T, Yoshida M, Ikeda T, Tominaga T. Concentration of vascular endothelial growth factor in the sera of normal controls and cancer patients. *Clin Cancer Res* 1996; **2**: 821-826
- 39 **Dirix LY**, Vermeulen PB, Pawinski A, Prove A, Benoy I, De Pooter C, Martin M, Van Oosterom AT. Elevated levels of the angiogenic cytokines basic fibroblast growth factor and vascular endothelial growth factor in sera of cancer patients. *Br J Cancer* 1997; **76**: 238-243
- 40 **Salven P**, Manpaa H, Orpana A, Alitalo K, Joensuu H. Serum vascular endothelial growth factor is often elevated in disseminated cancer. *Clin Cancer Res* 1997; **3**: 647-651
- 41 **Kraft A**, Weindel K, Ochs A, Marth C, Zmija J, Schumacher P, Unger C, Marme D, Gastl G. Vascular endothelial growth factor in the sera and effusions of patients with malignant and nonmalignant disease. *Cancer* 1999; **85**: 178-187
- 42 **Yoshiji H**, Kuriyama S, Yoshii J, Yamazaki M, Kikukawa M, Tsujinoue H, Nakatani T, Fukui H. Vascular endothelial growth factor tightly regulates *in vivo* development of murine hepatocellular carcinoma cells. *Hepatology* 1998; **28**: 1489-1496
- 43 **Sakamoto M**, Ino Y, Fujii T, Hirohashi S. Phenotype changes in tumor vessels associated with the progression of hepatocellular carcinoma. *Jpn J Clin Oncol* 1993; **23**: 98-104
- 44 **Terada T**, Nakanuma Y. Arterial elements and perisinusoidal cells in borderline hepatocellular nodules and small hepatocellular carcinomas. *Histopathology* 1995; **27**: 333-339
- 45 **Nakashima O**. Pathological diagnosis of hepatocellular carcinoma. *Nippon Rinsho* 2001; **59**(Suppl 6): 333-341

Edited by Wang XL Proofread by Zhu LH

# Salvage therapy for hepatocellular carcinoma with thalidomide

Tsang-En Wang, Chin-Roa Kao, Shee-Chan Lin, Wen-Hsiung Chang, Cheng-Hsin Chu, Johson Lin, Ruey-Kuen Hsieh

**Tsang-En Wang, Chin-Roa Kao, Shee-Chan Lin, Wen-Hsiung Chang, Cheng-Hsin Chu,** Gastroenterology Section, Department of Internal Medicine, Mackay Memorial Hospital, Taipei, Taiwan, China  
**Johson Lin, Ruey-Kuen Hsieh,** Oncology Section, Department of Internal Medicine, Mackay Memorial Hospital, Taipei, Taiwan, China  
**Correspondence to:** Dr. Tsang-En Wang, Gastroenterology Section, Department of Internal Medicine, Mackay Memorial Hospital and Mackay Junior College of Nursing, No.92 Section 2, Chang-San North Road, Taipei, Taiwan, China. tewang@ms2.mmh.org.tw  
**Telephone:** +886-2-25433535 **Fax:** +886-2-27752142  
**Received:** 2003-09-09 **Accepted:** 2003-11-06

## Abstract

**AIM:** To evaluate the clinical benefit of thalidomide in patients with advanced hepatocellular carcinoma (hepatoma).

**METHODS:** From March 2000 to July 2002, patients who had advanced hepatocellular carcinoma and failed to or were unsuited for aggressive treatment, were enrolled and took thalidomide 150 to 300 mg/d. All cases were followed till April 2003. Data collection included viral hepatitis, grade of cirrhosis, total dosage of thalidomide, side effect, stage of hepatoma by Okuda and CLIP classification, and prognosis. The subjects were divided into A and B groups, depending on 5 000 mg dosage of thalidomide. Survival time of all cases and in the two subgroups was evaluated.

**RESULTS:** Ninety-nine patients with hepatoma were enrolled, 81 men and 18 females with median age  $58 \pm 14.1$  years. Eighty-six percent had viral hepatitis and one case was alcoholism. Hepatoma was diagnosed with histology, alpha-fetoprotein (aFP)  $>400$  ng/mL, or image examination, there were 30, 33 and 36 cases respectively. At the time of thalidomide therapy, more than 81% had cirrhotic status. Twenty-two patients were in group A ( $<5$  000 mg) with median survival time about 25 days, for 77 cases in group B ( $\geq 5$  000 mg) the median survival time was about 109 days. Six subjects had partial response. Most adverse effects were skin rash, neuropathy, somnolence, and constipation.

**CONCLUSION:** Several patients responded to thalidomide therapy. As a single drug therapy, thalidomide might not have good therapeutic effect for all cases, but a small ratio of patients had exciting response, the resistance or tumor escape would develop after long-term use. Up to now, no defined facts could be used to predict response. The effect of thalidomide on hepatoma might be associated with the dosage. As salvage therapy, thalidomide has its value. Combination or adjuvant therapy will be the next trial.

Wang TE, Kao CR, Lin SC, Chang WH, Chu CH, Lin J, Hsieh RK. Salvage therapy for hepatocellular carcinoma with thalidomide. *World J Gastroenterol* 2004; 10(5): 649-653  
<http://www.wjgnet.com/1007-9327/10/649.asp>

## INTRODUCTION

Hepatocellular carcinoma (hepatoma) is a major cause of death in the world, especially in the endemic areas of viral hepatitis

B and C, such as Taiwan, China. Taiwan is a high prevalence area of hepatocellular carcinoma, more than 6 900 people died of hepatocellular carcinomas at Taiwan in 2002. Although the incidence rate of small hepatoma was increased in the last few years, but most patients had severe liver cirrhosis that made them lose the opportunities to receive curative therapy. Therapy of hepatocellular carcinoma in chronic liver disease patients is challenging also. Local treatments, such as surgical resection, ethanol intratumor injection, ablation with high frequency, or transhepatic artery embolization have been improved, but these procedure might cause new problems and the curative and survival rates of these patients are still low<sup>[1]</sup>. The average survival time is shorter than 6 months if metastasis occur. However, to seek new effective therapy for hepatoma, to prolong the patient life or improve their life quality with later stage of hepatoma are major issues at Taiwan.

Anti-angiogenesis is a new concept for cancer therapy, in the 1970's, Dr Folkman launched out the theory<sup>[2]</sup>. Neoplasm's growth depends on angiogenesis, angiogenesis inhibitors could block the process and treat neoplasm, especially vascularized ones. Many antiangiogenic agents are developed and some are going in clinical trials. Thalidomide is one of them and has been studied for its anti-angiogenic activity in last several years.

Hepatoma is a hypervascular tumor that has been proved by angiography and histology. For this reason, antiangiogenesis therapy may be effective for hepatoma. Up to now, few papers described the antiangiogenesis therapy for hepatoma. The first success case of hepatoma treated with thalidomide, was reported in 2000. A 67-year-old man had a 6 cm large tumor. Unfortunately, the tumor continued to grow after 5FU and interferon therapy, chemoembolization and chemotherapy. Thalidomide therapy was used. The tumor was shrunk, aFP was diminished, and the patient was alive in 2000<sup>[3]</sup>. Henceforward, some might had exciting results, some were disappointed. Although a few patients would get benefit from thalidomide therapy<sup>[4-8]</sup>. No final conclusion was made. In this study, we report our experience in using thalidomide as a salvage drug for the patients with hepatoma, who were unsuitable for other managements.

## MATERIALS AND METHODS

One hundred and six patients with hepatocellular carcinoma were entered into the study between Mar 2000 and July 2002. A diagnosis of hepatoma was made by histopathology, alpha fetoprotein (AFP) more than 400 ng/mL or image plus clinical manifestation. All of them were poor candidates for more aggressive treatment. Those patients were required to receive a risk-benefit counseling, to sign an informed-consent agreement, to use forms of birth control. The Institutional Ethics Committee of the Mackay Memorial Hospital and Department of Public Health in Taiwan approved the study protocol and the informed consent form.

They were given oral thalidomide table, containing 50 mg (Taiwan Tung Yang Biopharm Co. Ltd), at a dosage of 150 to 300 mg/day according to clinical reactions and adverse effects for a variable period. The drug was given 2 dosages in the morning and bedtime. The subjects were followed to April 2003. Ninety-nine patients were evaluated.

Depending the total dosage used, the patients were divided into two subgroups, group A took thalidomide less 100 tablets

(5 000 mg), and group B had more than 100 tablets. Data collected included viral hepatitis, grade of cirrhosis, total dosage of thalidomide, side effect, stage of hepatoma by Okuda and CLIP classification<sup>[9]</sup>, and prognosis. The trial was prompted by the observation of response and survival times in all case and in the two subgroups. Survival time was calculated with SPSS 10 software and observation time interval was one week.

## RESULTS

### General data

Ninety-nine patients were valuable for evaluation (Table 1). The ratio of men and women was 81:18. The median age of the patients was 58 years (range, 21-86 years, S=14.1 years). Fifty-eight patients were chronic hepatitis B, 22 patients had hepatitis C, and five patients were infected with viral hepatitis B and C. Six cases were confirmed with non-B and non-C hepatitis. Seven cases had an underminted condition. One was alcoholic cirrhosis without viral hepatitis. Among them, another 12 patients had alcohol consumption. At the time of hepatoma diagnosed, 81% (80 cases) of the patients had cirrhosis diagnosed with histology or image studies, 14 cases had chronic parenchyma disease, and only 5 patients had normal texture of liver. The hepatoma diagnosis was dependent on either histopathology, aFP >400 ng/mL or image and clinical manifestations. There were 33, 30 and 36 cases respectively. They were followed up, with median follow-up time about 6 months (average 177 days) to April 2003 in this study. Group A had 22 patients and group B had 77 patients.

**Table 1** Description of cases

Age (yr)	
Median	58±14.1
Range	21-86
Sex	
Male	81
Female	18
Modality of diagnosis	
Cytological/histological	33
Imaging + AFP > 400 ng/mL	30
Imaging + AFP < 400 ng/mL or unknown	36
Cirrhosis	
Absent	5
Present	80
Chronic parenchymaldis'	14
Causes of liver disease	
Hepatitis B	58
Hepatitis C	22
Hepatitis B +C	5
Non B and Non C	6
Alcoholic	1
Child-Pugh stage (unknown = 2)	
A	43
B	33
C	21
AFP (ng/mL) (unknown =10 )	
10 <	17
11-400	33
400	39
Portal vein thrombosis (unknown=3)	
No	46
Yes	50
Pre ThalidomideTreatment (unknown=7)	
No	30
Yes	72
Surgery	11
PEI	16
TACE	49
Radiation	6
Chemotherapy	3

### Laboratory data

The pretreatment median platelet count was  $187 \times 10^3/\text{mm}^3$ , leukocyte count was about  $6\,820/\text{mm}^3$ . Analyzed the liver function by Child' s classification, 43, 33 and 21 patients belong to grades A, B and C, respectively. Two cases were unclassified at the beginning of medication because of incomplete data record. Both of them were grade A when hepatoma was diagnosed. One had bone and lung metastasis and one was followed up at other hospital. Fifty-two percent of the patients had alpha-fetoprotein level more than 400 ng/mL. According to hepatoma stage Okuda (Table 2) and CLIP (Table 3), the numbers of patients and survival time in the both groups are shown in Table 4 and Table 5. There were no confirmed complete responses. Although no scheduled image evaluation was done, six patients having partial response were observed, yielding a rate of 7% at least. Four responded patients had a high serum aFP initially, which was decreased after treatment.

**Table 2** Okuda staging for HCC

Point	0	1
Size of tumor	<50% of liver	> 50 %
Ascites	No	Yes
Albumin	>=3	<3
Bilirubin	<3	>=3
Stage I: 0	II: 1 or 2	III: 3 or 4

**Table 3** CLIP scoring system

Scores Variables	0	1	2
Child-pugh stage	A	B	C
Tumor morphology	Uninodular and extension <=50%	Multinodular and extension <=50%	Massive or extension > 50%
AFP	<400	>=400	
Portal vein thrombosis	No	Yes	

**Table 4** Hepatocellular carcinoma stage and survival time, Okuda stage and survival time

Group		Stage			MSD
		I	II	III	
A (n=19)	Case No.	5	9	5	
	Survival days	161	26.8	10.5	25.2
B (n=76)	Case No.	19	41	16	
	Survival days	171.5	136.5	47.3	108.5

MSD: medium survival day.

**Table 5** Hepatocellular carcinoma stage and survival time, CLIP classification and survival time

Group		Score						
		0	1	2	3	4	5	6
A (n=20)	Case	0	2	2	2	8	5	1
	Survival days	220.5	49	42	35	12.3	11	
B (n=76)	Case	1	8	20	20	15	9	3
	Survival days	>345	301	150.5	106.8	96.2	59.5	19.3

### Response and survival

Overall, the median survival time was about 80 days to Apr 2003. Twenty-one of 22 patients in group A had expired, only one survived to Dec 2000 then lost follow-up. The median survival time in group A was 25 days. Most of them had a

poor condition for aggressive treatment and died due to liver function decompensation. Thirty-six percent patients of group A has Child-pugh's grade C, compared only 16% in group B. One third patients died with hepatic failure and 4 patients died with massive esophageal varices bleeding in group A. Fifty percent cases had multiple lesions in liver or distal metastasis. Some of them liked to try TAE again and other alternative therapies. One patient had grade 3 dermatologic toxicity. These were major causes the withdrawal of thalidomide. Seventy-seven patients were group B, 61 patients died, and 16 patients experienced disease progression or partial responses. The median overall survival time in group B was 108.5 days.

### Toxicity

The most common treatment-related toxic effects were skin itching, rash and urticaria. Twenty patients were relieved by antihistamine, two patients with severe dermatitis prompted discontinuation of thalidomide treatment. Other adverse effects included neuropathy, somnolence, and constipation and six had others side effect, such as gastrointestinal symptom. Most of them took laxative, therefore the rate of constipation was not real. Toxicity of thalidomide in our patients was tolerable.

**Table 6** Response rate of hepatoma treated with thalidomide

	Tumor response		Stabilization rate		
	N	CR/PR	SD	PD	(PR + SD)
Patt <i>et al.</i> '00 <sup>[6]</sup>	21	0/1	11	9	12 (57%)
Chen <i>et al.</i> '00 <sup>[8]</sup>	42	0/2	15	25	17 (43%)
Kong <i>et al.</i> '01 <sup>[5]</sup>	11	0/1	4	6	5 (45%)
Lin <i>et al.</i> '02 <sup>[4]</sup>	27	0/1	1	25	2 (8%)
Schwartz <i>et al.</i> '02 <sup>[7]</sup>	20	1/1	7	11	9 (45%)
Total	121	1/6	38	76	45 (37%)
Wang <i>et al.</i> '03	99	0/6	Survival 16 upto Apr 31 '03		

CR: complete response, PR: partial response, SD: stable disease, PD: Progressive disease.

### DISCUSSION

Thalidomide was developed in the 1950's and originally marketed as a sedative but was withdrawn after its teratogenic effects were recognized in 1964. FDA approved thalidomide for erythema nodosum leprosum in 1999, which stimulated new interests<sup>[10]</sup>. Thalidomide has been shown to be effective in treating cutaneous lupus erythematosus, idiopathic oral and oropharyngeal aphthous ulceration in HIV-1 positive patients. It has subsequently been used in the treatment of graft versus host disease, rheumatoid arthritis, inflammatory bowel disease, and the malignancy diseases in phase II or phase III, such as multiple myeloma, renal cell carcinoma, prostate cancer, breast cancer, ovary cancer, *etc.* The effects may come from its potent inhibitor of angiogenesis and immune response-modifying properties, which are essential to many physiologic and pathologic pathways. Thalidomide could inhibit the production of tumor necrosis factor- $\alpha$ <sup>[11,12]</sup> and alter multiple cytokines. Several other immunomodulatory effects have been reported, such as down-regulation of T-lymphocyte surface molecules, inhibition of lymphocyte proliferative responses to alloantigens and mitogens, and lowering of CD4:CD8 peripheral T-lymphocyte ratios<sup>[13,14]</sup>. It could induce a shift from T helper cell type 1(Th1) to Th2 T-cell responses and modify various cell surface receptors<sup>[15]</sup>.

Hepatoma is a hypervascular tumor, blood support comes from new branch vessels of hepatic artery. Presumably, chemoembolization intercept the vessels has become one of

standard treatments for hepatoma. We can speculate that anti-angiogenetic agents can inhibit hepatoma growth, such as thalidomide. Thalidomide was believed to be species-specific antiangiogenesis drug<sup>[16,17]</sup>. That is the most likely reason for its reported effectiveness against some solid tumors involving neoformation of blood vessels in early days. In addition, angiogenesis inhibitor TNP-470 can inhibit both the growth of primary tumors and the formation of liver metastases from gastric and colon cancer xenografts in nude mice. In a rat hepatoma model, it also enhanced apoptosis in hepatic metastases and improved survival<sup>[18]</sup>. Again, inhibition of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is one important action of thalidomide, which could be used to treat human hepatoma.

To treat hepatoma with thalidomide will be feasible. Some papers have actually discussed this issue since 1999, most were reported with case studies or in academic meetings<sup>[4-8]</sup> (Table 6). The complete response was rare, partial response rate was 5% to 10%, stable disease was about 37%, the variant was depending on the duration of observation, cancer stage of patients and definition of stability. In our study, no patient had complete remission, the initial partial response was 7%.

The effect of thalidomide on cancer is still mysterious. We surmised that interaction between the drug, patient immunity and heterogeneous cancer was very complex. The impact factors of response are multiple. However, there are some other antitumor mechanisms in addition to antiangiogenesis and TNF suppression. For example, degradation of tumor necrosis factor- $\alpha$  mRNA in human monocytes was modulated by thalidomide<sup>[12]</sup>. On the contrary, TNF- $\alpha$  production in IL-1 $\beta$ -stimulated or PMA-stimulated hepatocyte cultures was not altered following the addition of thalidomide<sup>[19]</sup>. Thalidomide can augment natural killer cell cytotoxicity. The number of NK cells increased in multiple myeloma after medication, but only those patients who responded to treatment showed an increase in the percentage of NK cells. Thalidomide for multiple myeloma might trigger the NK cells<sup>[20]</sup>. Same phenomena might occur in hepatoma. The exact mechanism of thalidomide is not fully clear, lot of effect wait to discover.

Why thalidomide for hepatoma does no work as a targeted therapy? Hepatoma was heterogeneous in genotype and phenotype<sup>[21]</sup>. Animal experiments showed that angioarchitecture and blood flow velocity in liver cancer were heterogeneous<sup>[22]</sup>. Adhesion molecular, E-cadherin, revealed various expressions among tumor samples<sup>[23]</sup>. These could explain why not all the solid tumors or patients had no response to thalidomide therapy, even in treatment of hepatoma. Although a few hepatomas had very good response to thalidomide beyond expectancy. At present, we still lack a landmark to select the suitable patients and specific parameters to predict the response to thalidomide treatment. Host may be another important factor affecting the response to thalidomide. All these need more researches to answer.

It seems that thalidomide may offer hepatocellular carcinoma stabilization, but has no significant antitumor activity. Most papers discussed the rate of response only. The survival time may be one of the major destinations in clinical trails of thalidomide therapy. A few phase I and II studies showed survival times slightly increased in hepatoma patient, but the survival time of those patients was also unpredictable and thalidomide did not significantly prolong the life of all patients. Our patients survived average of 25 and 108 days in groups A and B, respectively. The survival time appeared shorter than reported<sup>[9,24]</sup>. We thought the time was calculated from the day of thalidomide therapy, not the day of hepatoma diagnosed. In group A, the general condition of them was poor, they seemed not get any benefit from thalidomide therapy. All the response cases in our study were belong to group B with a large total dosage. Chen found the average survival time in patients with responded or stable disease was 269 days,

significantly longer than 74 days in the patients with progression<sup>[8]</sup>. Neben Kai reported the cumulative 3-month dosage was the remaining factor for overall survival rate in treating multiple myeloma<sup>[25]</sup>. At the same time, that might occur in HCC and the anticancer effect of thalidomide was associated with cell cycle and total dosage, as other chemotherapy. So, thalidomide might not be suitable for patients with too terminal status because they do not have chance to take enough dosage of thalidomide. It is the issue to be studied.

Besides, this study might have a worth finding. We usually think there is no drug resistance to antiangiogenetic therapy, but it did occur. We revealed a case with high aFP and lung metastasis had a good response. His serum level of aFP dropped and lung lesions became small and disappeared after thalidomide therapy. Unfortunately, the aFP increased again and lung lesion regrew after 9 months thalidomide continuous therapy. That means the resistant may develop if patients take thalidomide for enough long time. These need more case observations to confirm.

However, patients with hepatoma at later stage do not have time to wait for a new therapy. Most hepatomas develop in patients with cirrhosis. In this study, the ratio of cirrhosis was more than 80%. Lot of patients are poor candidates for aggressive treatment. Moreover, the prognosis of patients with metastatic or refractory HCC was very poor, to cure the patients was almost impossible. The aim of therapy was to improve their life quality and prolong their survival time<sup>[26]</sup>. Overall, as a single medication, thalidomide has the response rate about 5 to 10%.

At one time, thalidomide was notorious for its side effect that has been under control in last few years. Phocomelia is the most severe adverse effect that did not cause any problem in patients with hepatoma. The other adverse effects were sedation, constipation, and skin rash in our study. These effects were usually dose-related and mild, and could be treated except few patients who must discontinue medication. Peripheral axonal sensory neuropathy caused by thalidomide appeared only after a large dose accumulation. In our study, even at the later stage of cirrhosis, only few patients discontinued thalidomide. Another unusual adverse effect of thalidomide is vascular thrombosis. Deep venous thrombosis may occur, especially in patients with multiple myeloma and renal cell carcinoma, but has not seen in patients with hepatoma. Our results showed that thalidomide was tolerated in cirrhotic patients with hepatoma. Thalidomide is convenient for oral intake. It can be used as a salvage therapy before more powerful antiangiogenetics or other combined or adjuvant methods are developed. Several pilot studies have been on going<sup>[27]</sup>, such as combination therapy of thalidomide plus interferon alfa in phase II<sup>[28]</sup>. A combination of capecitabine and thalidomide, celecoxib and escalating doses of thalidomide in patients with unresectable HCC revealed few response patients<sup>[29,30]</sup>.

In conclusions, thalidomide is a drug with multifunctions, may be useful in some of patients with advanced hepatocellular carcinoma and it produces durable stability disease in approximately one third of patients, with a partial response rate not beyond 10%. Most side effects of thalidomide are minimal, it could be administered to patients with even significant liver cirrhosis and poor candidates for other therapies. However, thalidomide as a single drug formula for hepatoma is no so good, resistance will be appear after long-term use. If it is used as a salvage therapy in patients with hepatoma, some patients get benefit<sup>[27]</sup>. A controlled trial of thalidomide in selected patients with cirrhosis and hepatoma is warranted. The combination therapy with other drugs or adjuvant therapy in different stage hepatoma will be the next step studies. Thalidomide analogues have been under investigation. Hopefully, this will be the beginning of the development of a

new therapeutic modality for hepatoma.

## REFERENCES

- 1 **Lin SC**, Shih SC, Kao CR, Chou SY. Transcatheter arterial embolization treatment in patients with hepatocellular carcinoma and risk of pulmonary metastasis. *World J Gastroenterol* 2003; **9**: 1208-1211
- 2 **Folkman J**, Watson K, Ingber D, Hanahan D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature* 1989; **339**: 58-61
- 3 **Patt YZ**, Hassan MM, Lozano RD, Ellis LM, Peterson JA, Waugh KA. Durable clinical response of refractory hepatocellular carcinoma to orally administered thalidomide. *Am J Clin Oncol* 2000; **23**: 319-321
- 4 **Lin AY**, Brophy N, Fisher GA, So S, Biggs C, Yock T, Levitt L. Phase II study of thalidomide in patients (pts) with unresectable hepatocellular carcinoma (HCC). *Proc Am Soc Clin Oncol* 2002; **21**: p97b abstr:2202
- 5 **Kong HL**, Boyer MJ, Lim R, Clarke S, Milward JM, Wong E. Phase II trial of thalidomide in unresectable hepatocellular carcinoma (HCC)-a cancer therapeutics group (CTRG) study. *Proc Am Soc Clin Oncol* 2001; **20**: p133b, abstr: 2282
- 6 **Patt YZ**, Hassan MM, Lozano RD, Zeldis JB, Schnirer I, Frome A, Abbuzzese J, Wolff R, Brwom T, Lee E, Charnsangavej C. Phase II trial of thalidomide for treatment of nonresectable hepatocellular carcinoma. *Proc Am Soc Clin Oncol* 2000; **14**(Suppl 12, abstr): 1035
- 7 **Schwartz JD**, Sung Max W, Lehrer D, Goldenberg A, Muggia F, Volm M. Thalidomide for unresectable hepatocellular cancer (HCC) with optional interferon- $\gamma$  upon disease progression. *Proc Am Soc Clin Oncol* 2002 abstr: 1847
- 8 **Jacqueline WP**, Chen LT. Thalidomide and hepatoma. NHRI-AACR Joint Conference 2001 abstr: 653. (The Fifth Taiwan Cancer Clinical Research Organization Cooperative Annual Conference and Cross-Strait Anti-Cancer Pharmaceuticals Research and Development Conference.) <http://tpmd.nhri.org.tw/~scba/php-bin/scba2001/abstract/abs653.htm>
- 9 **The Cancer of the Liver Italian Program (CLIP) Investigators**. Prospective validation of the CLIP Score: a new prognostic system for patients with cirrhosis and hepatocellular carcinoma. *Hepatology* 2000; **31**: 840-845
- 10 **Cori V**. Preparing for thalidomide's comeback. *Ann Internl Med* 1997; **127**: 951-952
- 11 **Calabrese L**, Fleischer AB. Thalidomide current and potential clinical applications. *Am J Med* 2000; **108**: 487-495
- 12 **Moriera AL**, Sampaio EP, Zmuidzinas A, Frindt P, Smith KA, Kaplan G. Thalidomide exerts its inhibitory action on tumor necrosis factor [alpha] by enhancing mRNA degradation. *J Exp Med* 1993; **177**: 1675-1680
- 13 **Gad SM**, Shannon EJ, Krotoski WA, Hastings RC. Thalidomide induces imbalances in T-lymphocyte subpopulations in the circulating blood of healthy males. *Lepr Rev* 1985; **56**: 35-39
- 14 **Haslett PA**, Corral LG, Albert M, Kaplan G. Thalidomide costimulates primary human T lymphocytes, preferentially inducing proliferation, cytokine production, and cytotoxic responses in the CD8+subset. *J Exp Med* 1998; **187**: 1885-1892
- 15 **Verbon A**, Juffermans NP, Speelman P, van Deventer SJ, ten Berge IJ, Guchelaar HJ, van der Poll T. A single oral dose of thalidomide enhances the capacity of lymphocytes to secrete gamma interferon in healthy humans. *Antimicrob Agents Chemother* 2000; **44**: 2286-2290
- 16 **Bauer KS**, Dixon SC, Figg WD. Inhibition of angiogenesis by thalidomide requires metabolic activation, which is species-dependent. *Biochem Pharmacol* 1998; **55**: 1827-1834
- 17 **D'Amato RJ**, Loughnan MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. *Proc Natl Acad Sci U S A* 1994; **91**: 4082-4085
- 18 **Konno H**. Antitumor effect of angiogenesis inhibitor TNP-470 on human digestive organ malignancy. *Cancer Chemother Pharmacol* 1999; **43**(Suppl): S85-89
- 19 **Wordemann M**, Fandrey J, Jekmann W. Tumor necrosis factor-alpha production by human hepatoma cell lines is resistant to drugs that are inhibitory to macrophages. *J Interferon Cytokine*



- Res 1998; **18**: 1069-1075
- 20 **Davies FE**, Raje N, Hideshima T, Lentzsch S, Young G, Tai YT, Lin B, Podar K, Gupta D, Chauhan D, Treon SP, Richardson PG, Schlossman RL, Morgan GJ, Muller GW, Stirling DI, Anderson KC. Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. *Blood* 2001; **98**: 210-216
  - 21 **Hui AM**, Kawasaki S, Imamura H, Miyagawa S, Ishii K, Katsuyama T, Makuuchi M. Heterogeneity of DNA content in multiple synchronous hepatocellular carcinomas. *Br J Cancer* 1997; **76**: 335-339
  - 22 **Maksan SM**, Paulo H, Ryschich E, Kuntz C, Gebhard MM, Klar E, Schmidt J. *In vivo* assessment of angioarchitecture and micro-circulation in experimental liver cancer: a new model in rats. *Dig Dis Sci* 2003; **48**: 279-290
  - 23 **Wei Y**, Van Nhieu JT, Prigent S, Srivatanakul P, Tiollais P, Buendia MA. Altered expression of E-cadherin in hepatocellular carcinoma: correlations with genetic alterations, beta-catenin expression, and clinical features. *Hepatology* 2002; **36**: 692-701
  - 24 **Okuda K**, Ohtsuki T, Obata H, Tomimatsu M, Okazaki N, Hasegawa H, Nakajima Y, Ohnishi K. Nature history of hepatocellular carcinoma and prognosis in relation to treatment - study of 850 patients. *Cancer* 1985; **56**: 918-928
  - 25 **Neben K**, Moehler T, Benner A, Kraemer A, Egerer G, Ho AD, Goldschmidt H. Dose-dependent effect of thalidomide on overall survival in relapsed multiple myeloma. *Clin Cancer Res* 2002; **8**: 3377-3382
  - 26 **Feun LG**, Marini A, Molina E, O'Brien C, Schiff E, Jeffers L, Savaraj N. Thalidomide as palliative care for patients with unresectable hepatocellular carcinoma. *Proc Am Soc Clin Oncol* 2003; abstr: 865
  - 27 **von Moos R**, Stolz R, Cerny T, Gillesen S. Thalidomide; from tragedy to promise. *Swiss Med WKLY* 2003; **133**: 77-87
  - 28 **Schwartz JD**, Lehrer D, Mandeli J, Goldenberg A, Sung M, Volm M. Thalidomide in hepatocellular cancer (HCC) with optional interferon- $\alpha$  upon progression. *Proc Am Soc Clin Oncol* 2003; abstr: 1210
  - 29 **Chun HG**, Waheed F, Iqbal A, Wolf DC, Li Z, Kempin SJ. A combination of capecitabine and thalidomide in patients with unresectable, recurrent or metastatic hepatocellular carcinoma. *Proc Am Soc Clin Oncol* 2003; Abstr: 1407
  - 30 **Chen CS**, Hillebrand D, Hill K, Lilly M. A pilot study of celecoxib combined with escalating doses of thalidomide for treatment of unresectable hepatocellular carcinoma (HCC). *Proc Am Soc Clin Oncol* 2002; abstr: 2350

**Edited by** Wang XL **Proofread by** Xu FM

# Anti-liver cancer activity of TNF-related apoptosis-inducing ligand gene and its bystander effects

Chao He, Wei-Feng Lao, Xiao-Tong Hu, Xiang-Ming Xu, Jing Xu, Bing-Liang Fang

**Chao He, Wei-Feng Lao, Xiao-Tong Hu, Jing Xu,** Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, Hangzhou 310016, Zhejiang Province, China

**Xiang-Ming Xu,** First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

**Bing-Liang Fang,** Department of Thoracic and Cardiovascular Surgery, M.D. Anderson Cancer Center, Houston, 77030, USA

**Supported by** the National Natural Science Foundation of China, No. 30271467

**Correspondence to:** Dr. Chao He, Department of Oncology, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, Hangzhou 310016, Zhejiang Province, China. drhe@zju.edu.cn

**Telephone:** +86-571-86048962 **Fax:** +86-571-86993719

**Received:** 2003-03-20 **Accepted:** 2003-06-02

## Abstract

**AIM:** To observe the anti-liver cancer activity of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene and its bystander effects on hepatocellular carcinoma (HCC) cell line SMMC7721.

**METHODS:** Full-length cDNA of human TRAIL was transferred into SMMC7721 cells with a binary adenoviral vector system. Polymerase-chain reaction following reverse transcription (RT-PCR) was used to determine the expression of TRAIL gene. Effects of the transfected gene on proliferation of SMMC7721 cells were measured by MTT assay. Its influence on apoptosis was demonstrated by fluorescence-activated cell sorting (FACS). The bystander effect was observed by co-culturing the SMMC7721 cells with and without the transfected TRAIL gene at different ratios, and the culture medium supernatant from the transfected cells was also examined for its influence on SMMC7721 cells.

**RESULTS:** The growth-inhibition rate and apoptotic cell fraction in the cells transfected with the TRAIL gene, Bax gene or only LacZ gene were 91.2%, 48.0%, 28.8% and 29.1%, 12.5%, 6.6%, respectively. The growth-inhibition rate of transfection with these three sequences in normal human fibroblasts was 6.1%, 45.5% and 7.6%, respectively, indicating a discriminative inhibition of TRAIL transfection on the cancer cells. In the co-culturing test, addition of the transfected TRAIL to SMMC7721 cells in proportions of 5%, 25%, 50%, 75% and 100%, resulted in a growth-inhibition of 15.9%, 67%, 80.2%, 86.4% and 87.7%, respectively. We failed to observe a significant growth-inhibition effect of the culture medium supernatant on SMMC7721 cells.

**CONCLUSION:** TRAIL gene transferred by a binary adenoviral vector system can inhibit proliferation of SMMC7721 cells and induce their apoptosis. A bystander effect was observed, which seemed not to be mediated by soluble factors.

He C, Lao WF, Hu XT, Xu XM, Xu J, Fang BL. Anti-liver cancer activity of TNF-related apoptosis-inducing ligand gene and its bystander effects. *World J Gastroenterol* 2004; 10(5): 654-659 <http://www.wjgnet.com/1007-9327/10/654.asp>

## INTRODUCTION

TRAIL, first identified by searching an expressed sequence tag (EST) database with a conserved sequence contained in many tumor necrosis factor (TNF) family members, appears to induce apoptotic cell death only in tumorigenic or transformed cells and not in most of normal cells<sup>[1,2]</sup>. TRAIL has five receptors, including two death receptors DR4 and DR5, two decoy receptors DcR1 and DcR2, one soluble receptor osteoprotegerin. TRAIL is expressed constitutively in many normal tissues, which suggests that normal cells contain mechanisms that protect them from apoptosis induced by TRAIL. One explanation reported was that the decoy receptors DcR1 and DcR2 and another receptor osteoprotegerin could compete with DR4 and DR5 for TRAIL binding<sup>[3,4]</sup>. Furthermore, TRAIL has a synergistic effect with chemotherapeutic drugs to kill tumor cells and cause substantial tumor regression<sup>[5-7]</sup>. Much evidence have shown that repeated intravenous injection of a recombinant, biologically active TRAIL protein could induce tumor cell apoptosis, suppress tumor progression, and improve the survival of animals bearing solid tumors without any detectable toxicity in nonhuman primates<sup>[5,8]</sup>. Therefore, it appears that TRAIL may act as a potent anticancer agent. Furthermore, TRAIL can elicit apoptotic bystander effects on malignant cells. However, few researches of anti-liver cancer activity and bystander effects of TRAIL have been carried out up to date. Here, we first transferred human TRAIL gene to liver cancer cell line SMMC7721 with a binary adenoviral vector system, and assessed the anti-liver cancer activity of TRAIL gene and explored its bystander effects. In addition, we also assessed the toxicity of TRAIL to normal human fibroblasts (NHFB).

## MATERIALS AND METHODS

### Cell lines and culture conditions

Human embryonal kidney cells transformed by introducing sheared fragments of Ad5 DNA (293 cell) and human liver cancer cell line SMMC7721 were obtained from the Key Laboratory of Infective Diseases under Ministry of Public Health (Zhejiang University), normal human fibroblasts (NHFB) from normal human bone marrow were cultured; Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (NHFB with 20% fetal calf serum) in a 5% CO<sub>2</sub> atmosphere at 37 °C.

### Adenoviral vectors

Adenoviral vectors Ad/GT-Bax, Ad/GT-LacZ and Ad/PGK-GV16 were constructed as described previously<sup>[9,10]</sup>. Ad/GT-TRAIL, an adenoviral vector expressing TRAIL, was also constructed as described previously<sup>[9]</sup>. Amplification, titration, and quality analysis of all of the vectors were performed as described previously<sup>[9,10]</sup>. The titer determined by the absorbency of dissociated viruses at A<sub>260 nm</sub> (one A<sub>260 nm</sub> unit=10<sup>12</sup> viral particles/ml) was used in the study, whereas the titers determined by plaque assay were used as additive information.

### Transgene expression of TRAIL

As determined in preliminary experiments, cells were

coinfecting with Ad/GT-TRAIL or Ad/GT-LacZ and Ad/PGK-GV16 at a ratio of 1:1. The optimal MOI was determined by infecting each cell line with Ad/GT-LacZ + Ad/PGK-GV16 and the expression of  $\beta$ -galactosidase was assessed via X-gal staining. The MOI that resulted in >80% of blue stained cells were used in this experiment. These MOI were 1000 particles for SMMC7721 and NHFB. Unless otherwise specified, Ad/GT-LacZ and Ad/PGK-GV16 were used as the vector control for Ad/GT-TRAIL and Ad/PGK-GV16. Ad/GT-Bax and Ad/PGK-GV16 were used as the positive control. Cells treated with PBS only were used as a blank control.

$1 \times 10^6$  SMMC7721 cells were plated on 6-well plates and infected with Ad/GT-TRAIL+ Ad/PGK-GV16 or Ad/GT-LacZ + Ad/PGK-GV16. Forty-eight hours after infection, the cells were harvested and washed in PBS. RNA was extracted from the cells using Trizol reagent (Life Technology Inc.) and reversely transcribed to cDNA. The PCR conditions for cDNA amplification were 35 cycles of at 95 °C for 45 s, at 58 °C for 45 s, at 72 °C for 45 s, forward primer: 5' -AGA CCT GCG TGC TGA TCG TG-3', and reverse primer: 5' -TTA TTT TGC GGC CCA GAG CC-3'. The PCR products were separated in a 10g/L agarose gel and visualized by ethidium bromide staining.

### Cell viability

Cell viability was assessed using MTT assay (Amresco) according to the manufacturer's protocol. The  $5 \times 10^3$  SMMC7721 and NHFB cells were inoculated in to 96-well plates, with 3 parallel teams. Twenty-four hours after inoculation, the cells were infected with Ad/GT-TRAIL+ Ad/PGK-GV16, Ad/GT-Bax+ Ad/PGK-GV16, Ad/GT-LacZ + Ad/PGK-GV16 at MOI of 1000, and treated with PBS. At the 1<sup>st</sup>, 3<sup>rd</sup> and 5<sup>th</sup> day after infection, the cells were incubated with 5mL/L MTT for 4 h. Then the medium was removed and 150  $\mu$ L of sterilized DMSO solution was added, followed by incubation at 37 °C for 4 h. The absorbance of the reaction solution at 490 nm was measured. These data were used to make growth curves. The cell growth inhibition rate was (1-absorbance of experimental group/absorbance of control group) $\times 100\%$ .

### Apoptosis

Cell apoptosis was assessed by observing morphology and using the Annexin Vkit (Immunotech, Annexin-FITC) according to the manufacturer's protocol. The  $5 \times 10^4$  SMMC7721 cells were inoculated in to 6-well plates. Twenty-four hours after inoculation, the cells were infected with adenoviruses at MOI of 1000. Then the cell morphology was observed with a reversed microscope every day. On the 4<sup>th</sup> day, the cells were harvested by trypsinization, washed in PBS and labeled with ANNEXIN V and propidium iodide (PI) according to the manufacturer's protocol. Finally, they were subjected to flow cytometry to determine the extent of cell death.

### Bystander effects

Bystander effects of the TRAIL gene were assayed by MTT as follows:  $5 \times 10^4$  SMMC7721 cells were washed in PBS and plated on 80-mm dishes, cultured with fresh RPMI 1640. Twenty-four hours later, the cells were infected with adenoviruses at MOI of 1000. Another 24 h after infection, the cells were harvested as the transferred SMMC7721 cells (SMMC7721/TRAIL cells). SMMC7721/TRAIL and SMMC7721 cells were suspended in  $2 \times 10^4$ /ml of RPMI 1640 medium. SMMC7721/TRAIL and SMMC7721 cells were mixed with different ratios, SMMC7721/TRAIL cells accounted for 0, 5%, 25%, 50%, 75% and 100%, respectively.

Mixed cells ( $1 \times 10^4$ ) were inoculated in to 96-well plates with 3 parallel teams each ratio. Four days later the cell viability was determined by MTT assay.

### Mechanism of bystander effects

In this study we tried to discover the effect of soluble factors on bystander effects of TRAIL. The  $1 \times 10^6$  SMMC7721 cells were washed in PBS and plated on 80-mm dishes, cultured with fresh RPMI 1640. Twenty-four hours later, the cells were infected with Ad/GT-TRAIL+ Ad/PGK-GV16, and then cultured for another 24 h. Finally, the dishes were sent to be centrifuged and the medium was collected. This medium was filtrated with a 0.22  $\mu$ m filter membrane. Other dishes of non-infected SMMC7721 cells were cultured with this filtrated medium. We made the RPMI 1640 medium as blank control and Ad/GT-TRAIL+ Ad/PGK-GV16 as positive control. Four days later, the cell viability was assessed with MTT assay.

### Statistical analysis

Statistical analysis was performed with SPSS 10.0, the cells viability and the cell apoptosis ratio were determined by paired *t*-test. Statistical significance was set when  $P < 0.05$ .

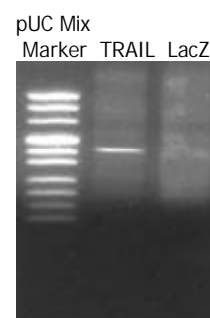
## RESULTS

### Virus titers

The titers of viruses determined by  $A_{260}$  were  $1 \times 10^{10}$  particles/mL.

### Transgene expression of TRAIL

The expression of TRAIL gene with this system was confirmed *in vitro* in human liver cancer cell line SMMC7721 by RT-PCR. Treatment of cells with Ad/GT-TRAIL+ Ad/PGK-GV16 resulted in a strong TRAIL-specific band, whereas infection with control vectors resulted in undetectable expression (Figure 1). It indicated that TRAIL gene was transferred into the SMMC7721 cells and the binary adenoviral vectors were effective.



**Figure 1** Expression of TRAIL tested with RT-PCR assay.

### Cell viability

In cultured SMMC7721 cells, the cell viability (MTT) study showed a significant difference in cell killing effects between lines responsive to treatment with TRAIL-expression vectors versus control vectors, blank controls, even positive controls (Table 1, Figure 2). In cultured NHFB cells, the cell viability study showed a significant difference in cell killing only between TRAIL-expression vectors and Bax-expression vectors (Table 2, Figure 3). These results demonstrated that treatment with TRAIL gene could effectively elicit cell killing in cultured human liver cancer cells but not in normal human fibroblasts. TRAIL gene was more effective than Bax gene in killing cultured human liver cancer cells. Bax gene was obviously toxic to normal human fibroblasts.

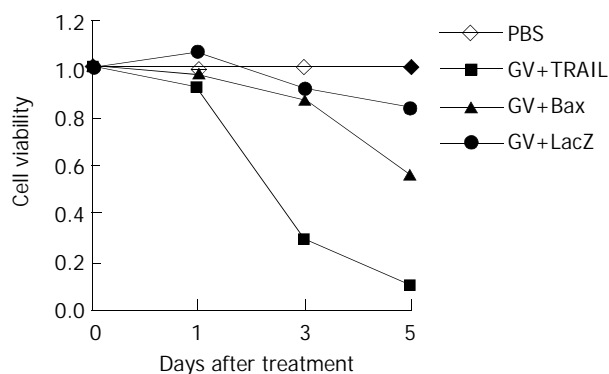
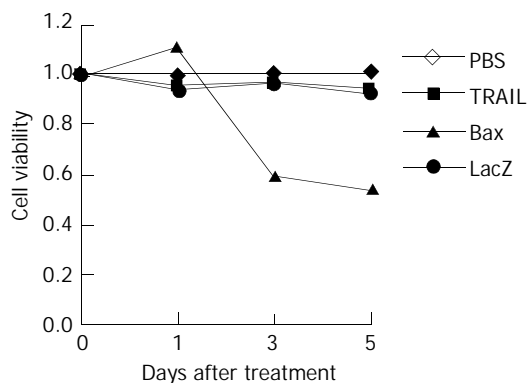
**Table 1** Cell growth-inhibition of SMMC7721 cells

Groups	OD(mean±SD)	Cell growth-inhibition rate (%)
PBS	1.25±0.20	0
TRAIL	0.11±0.02 <sup>ac</sup>	91.2
Bax	0.65±0.13 <sup>a</sup>	48.0
LacZ	0.89±0.04	28.8

<sup>a</sup>*P*<0.05 vs PBS, <sup>c</sup>*P*<0.05 vs Bax.

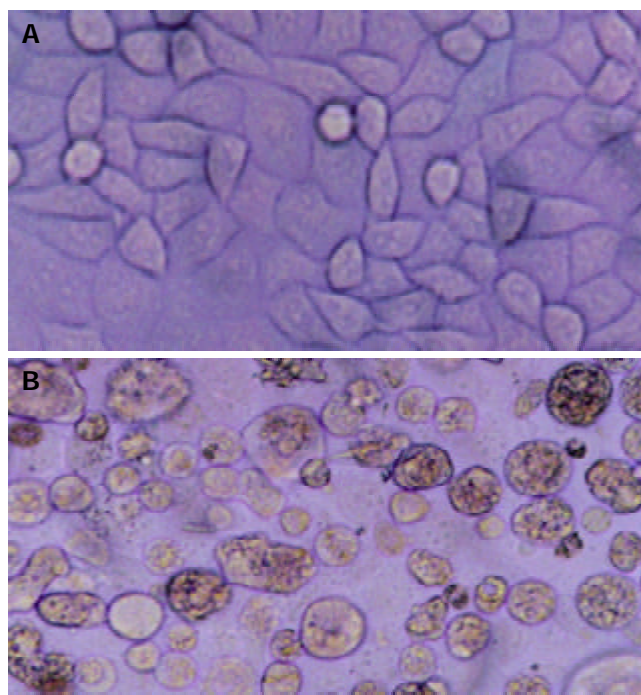
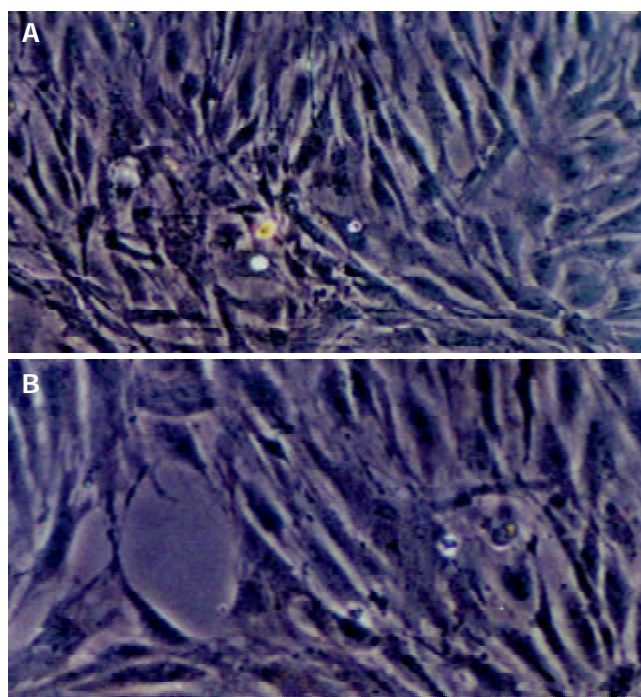
**Table 2** Cell growth-inhibition of NHFB cells

Groups	OD(mean±SD)	Cell growth-inhibition rate (%)
PBS	0.66±0.02	0
TRAIL	0.62±0.02 <sup>c</sup>	6.1
Bax	0.36±0.02 <sup>a</sup>	45.5
LacZ	0.61±0.03	7.6

<sup>a</sup>*P*<0.05 vs PBS, <sup>c</sup>*P*<0.05 vs Bax.

**Figure 2** Viability of SMMC7721 cells.

**Figure 3** Viability of NHFB cells.

### Apoptosis

Morphologic changes of SMMC7721 cells showed cell apoptosis in cells treated with TRAIL gene versus PBS (Figure 4). NHFB cells treated with TRAIL gene and PBS showed no obvious difference in morphology (Figure 5). Assessed by FCM, the apoptosis of SMMC7721 cells infected with TRAIL gene, Bax gene, LacZ gene and treated with PBS was 29.07%, 12.53%, 6.58%, and 2.94%, respectively. There were significant differences between SMMC7721 cells treated with TRAIL gene and Bax gene, LacZ gene and PBS (Table 3, Figure 6). It indicated that TRAIL gene had a great ability to induce apoptosis of human liver cancer cells. It was also shown that TRAIL gene was more effective than Bax gene in inducing apoptosis of human liver cancer cells.


**Figure 4** SMMC7721 cells. A: uninfected with Ad/GT-TRAIL+Ad/PGK-GV16, B: infected with Ad/GT-TRAIL+Ad/PGK-GV16.

**Figure 5** NHFB cells. A: uninfected with Ad/GT-TRAIL+Ad/PGK-GV16, B: infected with Ad/GT-TRAIL+Ad/PGK-GV16.

### Bystander effects

The results of MTT assay showed that when transduced SMMC7721/TRAIL cells accounted for 5%, 25%, 50%, 75% and 100% of all cells, 4 days later 15.9%, 67.0%, 80.2%, 86.4%, 87.7% of all cells were killed (Table 4). It showed that partial untransfected cells were killed by bystander effects of TRAIL gene.

Moreover, we found that bystander effects could not be transferred by the medium leached transduced cell components. The viability of SMMC7721 cells cultured with the medium leached transduced cell components was 96%, similar to that

cultured with fresh RPMI 1640 (which was set to be 100%). There were no significant differences between them ( $P>0.05$ , Table 5).

**Table 3** Percentage of apoptotic SMMC7721 cells

Groups	Percentage of apoptotic cells (%), mean $\pm$ SD
TRAIL	29.07 $\pm$ 4.96 <sup>ace</sup>
Bax	12.53 $\pm$ 1.23 <sup>ae</sup>
LacZ	6.58 $\pm$ 0.49 <sup>ac</sup>
PBS	2.94 $\pm$ 0.63 <sup>ce</sup>

<sup>a</sup> $P<0.05$  vs PBS, <sup>c</sup> $P<0.05$  vs Bax, <sup>e</sup> $P<0.05$  vs LacZ.

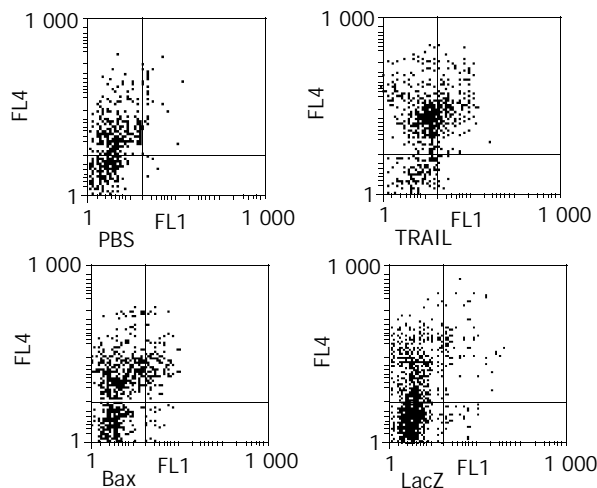
**Table 4** Bystander effects of TRAIL on SMMC7721 cells

Percentage of SMMC7721/TRAIL(%)	OD (mean $\pm$ SD)	Cell growth-inhibition rate (%)
0	0.693 $\pm$ 0.028	0
5	0.583 $\pm$ 0.036	15.9
25	0.228 $\pm$ 0.014	67.0
50	0.137 $\pm$ 0.019	80.2
75	0.094 $\pm$ 0.009	86.4
100	0.084 $\pm$ 0.010	87.7

**Table 5** Percentage of apoptotic SMMC7721 cells

Groups	OD (mean $\pm$ SD)	Cell growth-inhibition rate (%)
PBS	0.846 $\pm$ 0.016	0
Cultured medium	0.794 $\pm$ 0.027	4.0
TRAIL	0.108 $\pm$ 0.010 <sup>a</sup>	87.5

<sup>a</sup> $P<0.05$  vs PBS.



**Figure 6** SMMC7721 cell apoptosis.

## DISCUSSION

Liver cancer is one of the most malignant cancers with poor prognosis, and about two thirds of patients have been in China<sup>[11]</sup>. Though many therapeutic methods have been available to its treatment, no one showed a more notable effect than operation. However, the patients who could be radically operated were less than 15%<sup>[11]</sup>. It is now considered that apoptosis plays an important role in tumorigenesis, and at the same time inducing apoptosis of malignant cells has been prevailing in tumor therapy. There have been clinical reports of primary hepatocellular carcinoma treated with wild-type

p53 gene<sup>[12]</sup>.

TRAIL has become an attractive molecule for the treatment of cancer because it could kill tumor cells<sup>[5,8]</sup>. Studies of recombinant TRAIL protein have revealed that the extracellular portion of TRAIL molecule was sufficient for its antitumor activity but its homotrimerization was necessary for TRAIL protein to retain this activity<sup>[5,8]</sup>, suggesting that the conformational structure of TRAIL is crucial for interaction with its receptors. In this study, we studied whether TRAIL gene could be directly transferred into tumor cells, and whether the expression of biologically active molecules could effectively kill human liver cancer cell lines rather than normal human cells *in vitro*, and whether the bystander effect exists during the process of killing.

It has been reported that a variety of malignant tumors, such as breast carcinoma<sup>[13]</sup>, thyroid carcinoma<sup>[14]</sup>, melanoma<sup>[15]</sup>, glioma<sup>[16]</sup>, multiple myeloma<sup>[17,18]</sup>, colon carcinoma<sup>[19]</sup> and pancreatic carcinoma<sup>[20,21]</sup> were all sensitive to TRAIL, but few reports related to liver cancers were available. In this study, binary adenoviral vectors were used to introduce the therapeutic genes into liver cancer cell line SMMC7721 and normal human fibroblasts (NHBF) to evaluate their anti-tumor activity, toxicity and bystander effects.

It was difficult to design an adenoviral vector to transexpress proapoptotic genes because of their high proapoptotic activity and toxicity on packaging 293 cells. Arai *et al.*<sup>[22]</sup> reported that they constructed an adenovirus vector containing Fas-L gene, but it required packaging 293 cells bearing resistance to Fas-L or was decorated with Caspase depressor. Okuyama *et al.*<sup>[23]</sup> designed another system to achieve the transexpression of proapoptotic genes but it was too complicated. We constructed a binary adenovirus vector system, which showed that TRAIL gene had a strong proapoptotic activity on packaging 293 cells. On the other hand this system could augment transgene expressions via a GAL4 gene regulatory system. In brief, we constructed two mated vectors, Ad/PGK-GV16 and Ad/GT-TRAIL, the promoter GT was a synthetic promoter consisting of five GAL4-binding sites and a TATA box, which had a very low transcriptional activity *in vitro* and *in vivo* when it was placed in an adenoviral backbone. But the transgene activity could be substantially induced *in vitro* and *in vivo* by administering this construct in combination with an adenoviral vector (Ad/PGK- GV16) expressing a GT transactivator, the GAL4-VP16 fusion protein<sup>[24,25]</sup>. A weak promoter PGK would drive expression of the GAL4/VP16 fusion protein (GV16), which in turn would transactivate a minimal synthetic promoter, GAL4/TATA (GT), upstream of a transgene. It has been reported that this system used in CEA-positive cells treated with Ad/CEA-GV16+Ad/GT-LacZ versus Ad/CEA-LacZ had a 20- to 100- fold increase in transgene expression<sup>[24]</sup>. We determined the ratio of two vectors as 1:1 via X-gal staining with LacZ gene expression. The binary adenoviral vector system was effective for expressing high-level products of the proapoptotic gene. This has been confirmed in Bax gene study<sup>[5,26]</sup>, therefore, Bax gene was used as a positive control in our study. We detected the transgene expression of TRAIL gene by RT-PCR and the result showed quite positive. Another result was that proapoptotic genes TRAIL and Bax had a high toxicity to liver cancer cells while LacZ did not by using this system.

TRAIL demonstrated its antitumor activity in a variety of tumors<sup>[1,2,5]</sup>, although different tumors might vary in their sensitivity. This phenomenon was observed in this study as before<sup>[27]</sup>. SMMC7721 cells showed 29% of apoptosis. In comparison with other cell lines we tested before, HT29 had 24.6% of apoptosis<sup>[28]</sup>, DLD-1, H460 and A549 had 77%, 26% and 43.5% of apoptosis<sup>[27]</sup>, respectively. The mechanism underlying the differential sensitivity of malignant cells to

TRAIL treatment, as well as the differential killing of normal cells versus malignant cells, remain to be delineated. It could be partially explained by the presence of multiple receptors for TRAIL that functioned as either death-inducing or decoy receptors<sup>[4,29,30]</sup>. Some groups have proposed that expression of decoy receptors confer resistance to normal tissues<sup>[29,30]</sup>, others have suggested that the level of intracellular Caspase/apoptosis inhibitors including FLIP and Bcl-X<sub>L</sub> might result in resistance<sup>[15,31,32]</sup>. Oppositely, some other studies considered that the levels of DR4, DR5, and DcR1 could not explain this phenomenon<sup>[7,15,27,31]</sup>. Accordingly, we hold that the presence of decoy receptors is not the exclusive reason for different sensitivity of different cells. Recently, it was reported that a combined treatment with certain chemotherapy reagents could sensitize resistance to TRAIL-induced apoptosis<sup>[5,6,33]</sup>. But one of these reports also mentioned that normal cells could be sensitized to TRAIL-inducing apoptosis<sup>[7]</sup>, suggesting that such a combination treatment may also increase toxicity.

Our study revealed that treatment with TRAIL gene was nontoxic to normal human fibroblasts cultured *in vitro*. This result was consistent with previous reports<sup>[5,8,27]</sup>. Recently it was reported that human hepatocytes were very sensitive to apoptosis induced by recombinant TRAIL protein<sup>[34]</sup>. But the toxicity could arise from histamine or/and leucine taken in combination with recombinant TRAIL protein<sup>[35,36]</sup>. Another report showed that Z-LEHD-FMK, one of the Caspase inhibitors, could protect hepatocytes from apoptosis induced by TRAIL, but TRAIL gene could induce apoptosis of malignant cells<sup>[37]</sup>. Now, we can transfer transgenes directly into target cells by target techniques, such as tumor specific promoter hTERT<sup>[38]</sup>, to reduce the toxicity to normal cells.

One of the bottlenecks of gene therapy for malignant tumors is the low transgene efficiency. It is difficult to transfer target genes into each tumor cell. Investigators have attempted to circumvent this limitation by exploiting what was called the bystander effects based on the transfer of vector transgene products from infected to uninfected cells. Though the existence of bystander effects could not increase the transgene efficiency, it could enhance the cell killing capability. Theoretically, treatment with TRAIL may elicit bystander effects either through interaction of cell surface TRAIL molecules with receptors on the neighboring cells or through the action of soluble TRAIL from TRAIL-expression cells. It has been reported that TRAIL gene could exert proapoptotic bystander effects on cancer cells<sup>[27,39]</sup> as we demonstrated.

TRAIL is a type II membranous protein, it is speculated that membrane-bound TRAIL can be cleaved and then turned into a soluble form. Both membrane-bound TRAIL and soluble TRAIL could rapidly induce apoptosis in a wide variety of tumor cell lines via interaction with the death receptors DR4 and DR5<sup>[40,41]</sup>. Nevertheless, in our study, the cell killing effects of TRAIL gene were not transferable with the medium of TRAIL-expressing cell cultures. This result suggested that the proapoptotic activity of TRAIL gene was mainly elicited via membrane-bound TRAIL. The soluble factors contributed little to antitumor activity and to the bystander effects of TRAIL gene. One explanation is that the effects of soluble TRAIL may be dose-dependent. In previous reports, the cell killing activity of recombinant soluble TRAIL was demonstrated, but the effect was elicited by high doses of TRAIL. They could not be achieved by spontaneous cleavage of TRAIL from cultured cells.

In conclusion, we found that TRAIL gene was more effective than Bax gene in killing liver cancer cells with bystander effects. TRAIL had no toxicity to normal cells. These results suggest that TRAIL gene is more effective as a therapeutic gene of malignant tumors than Bax gene.

More researches should be carried out to reveal the anti-

liver cancer activity and bystander effects of TRAIL *in vivo*. It is possible that TRAIL gene will be used in clinical practices as new promoters and neoteric transgene vector systems are developed.

## REFERENCES

- 1 **Wiley SR**, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C, Smith CA. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 1995; **3**: 673-682
- 2 **Pitti RM**, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* 1996; **271**: 12687-12690
- 3 **Ashkenazi A**, Dixit VM. Apoptosis control by death and decoy receptors. *Curr Opin Cell Biol* 1999; **11**: 255-260
- 4 **Griffith TS**, Lynch DH. TRAIL: a molecule with multiple receptors and control mechanism. *Curr Opin Immunol* 1998; **10**: 559-563
- 5 **Ashkenazi A**, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokh Z, Schwall RH. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* 1999; **104**: 155-162
- 6 **Lacour S**, Micheau O, Hammann A, Drouineaud V, Tschopp J, Solary E, Dimanche-Boitrel MT. Chemotherapy enhances TNF-related apoptosis-inducing ligand DISC assembly in HT29 human colon cancer cells. *Oncogene* 2003; **22**: 1807-1816
- 7 **Keane MM**, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. Chemotherapy augments TRAIL-induced apoptosis in breast cell line. *Cancer Res* 1999; **59**: 734-741
- 8 **Walczak H**, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC, Lynch DH. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. *Nat Med* 1999; **5**: 157-163
- 9 **Fang BL**, Ji L, Bouvet M, Roth JA. Evaluation of GAL4/TATA *in vivo* induction of transgene expression by adenovirally mediated gene codelivery. *J Biol Chem* 1998; **273**: 4972-4975
- 10 **Kagawa S**, Gu J, Swisher SG, Lin J, Roth JA, Lai D, Stephens LC, Fang B. Antitumor effect of adenovirus-mediated Bax gene transfer on p53-sensitive and p53-resistant cancer lines. *Cancer Res* 2000; **60**: 1157-1161
- 11 **Wu MC**. Clinical research advances in primary liver cancer. *World J Gastroenterol* 1998; **4**: 471-474
- 12 **Habib NA**, Ding SF, Masry R, Mitry RR, Honda K, Michail NE, Dalla Serra G, Izzi G, Greco L, Bassyouni M, el-Toukhy M, Abdel-Gaffar Y. Preliminary report: the short term effects of direct p53 DNA injection in primary hepatocellular carcinomas. *Cancer Detect Prev* 1996; **20**: 103-107
- 13 **Lin T**, Huang X, Gu J, Zhang L, Roth JA, Xiong M, Curley SA, Yu Y, Hunt KK, Fang B. Long-term tumor-free survival from treatment with the GFP-TRAIL fusion gene expressed from the hTERT promoter in breast cancer cells. *Oncogene* 2002; **21**: 8020-8028
- 14 **Ahmad M**, Shi Y. TRAIL-inducing apoptosis of thyroid cancer cells: potential for therapeutic intervention. *Oncogene* 2000; **19**: 3363-3371
- 15 **Griffith TS**, Chin WA, Jackson GC, Lynch DH, Kubin MZ. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. *J Immunol* 1998; **161**: 2833-2840
- 16 **Knight MJ**, Riffkin CD, Muscat AM, Ashley DM, Hawkins CJ. Analysis of FasL and TRAIL induced apoptosis pathways in glioma cells. *Oncogene* 2001; **20**: 5789-5798
- 17 **Lincz LF**, Yeh TX, Spencer A. TRAIL-induced eradication of primary tumour cells from multiple myeloma patient bone marrows is not related to TRAIL receptor expression or prior chemotherapy. *Leukemia* 2001; **15**: 1650-1657
- 18 **Chen Q**, Gong B, Mahmoud-Ahmed AS, Zhou A, Hsi ED, Hussein M, Almasan A. Apo2L/TRAIL and Bcl-2-related proteins regulate type I interferon-induced apoptosis in multiple myeloma. *Blood* 2001; **98**: 2183-2192
- 19 **Burns TF**, El-Deiry WS. Identification of inhibitors of TRAIL-induced death (ITIDs) in the TRAIL-sensitive colon carcinoma



- cell line SW480 using a genetic approach. *J Biol Chem* 2001; **276**: 37879-37886
- 20 **Trauzold A**, Wermann H, Arlt A, Schutze S, Schafer H, Oestern S, Roder C, Ungefroren H, Lampe E, Heinrich M, Walczak H, Kalthoff H. CD95 and TRAIL receptor-mediated activation of protein kinase C and NF-kappaB contributes to apoptosis resistance in ductal pancreatic adenocarcinoma cells. *Oncogene* 2001; **20**: 4258-4269
  - 21 **Ibrahim SM**, Ringel J, Schmidt C, Ringel B, Muller P, Koczan D, Thiesen HJ, Lohr M. Pancreatic adenocarcinoma cell lines show variable susceptibility to TRAIL-mediated cell death. *Pancreas* 2001; **23**: 72-79
  - 22 **Arai H**, Gordon D, Nabel EG, Nabel GJ. Gene transfer of Fas ligand induces tumor regression *in vivo*. *Proc Natl Acad Sci U S A* 1997; **94**: 13862-13867
  - 23 **Okuyama T**, Fujino M, Li XK, Funeshima N, Kosuga M, Saito I, Suzuki S, Yamada M. Efficient Fas-ligand gene expression in rodent liver after intravenous injection of a recombinant adenovirus by the use of a Cre-mediated switching system. *Gene Ther* 1998; **5**: 1047-1053
  - 24 **Koch PE**, Guo ZS, Kagawa S, Gu J, Roth RA, Fang B. Augmenting transgene expression from carcinoembryonic antigen (CEA) promoter via a GAL4 gene regulatory system. *Mol Ther* 2001; **3**: 278-283
  - 25 **Pan G**, Ni J, Yu G, Wei YF, Dixit VM. TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signaling. *FEBS Lett* 1998; **424**: 41-45
  - 26 **He C**, Xu XM, Hu XT, Fang BL. Experimental study of effects of bax gene on human colorectal cancer cell line HT-29. *Zhonghua Xiaohua Zazhi* 2002; **22**: 535-538
  - 27 **Kagawa S**, He C, Gu J, Koch P, Rha SJ, Roth JA, Curley SA, Stephens LC, Fang B. Antitumor activity and bystander effects of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene. *Cancer Res* 2001; **61**: 3330-3338
  - 28 **Xu XM**, He C, Hu XT, Fang BL. Tumor necrosis factor-related apoptosis-inducing ligand gene on human colorectal cancer cell line HT29. *World J Gastroenterol* 2003; **9**: 965-969
  - 29 **Pan G**, Ni J, Wei YF, Yu G, Gentz R, Dixit VM. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* 1997; **277**: 815-818
  - 30 **Sheridan JP**, Masters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, Ramakrishnan L, Grey CL, Baker K, Wood WI, Goddard AD, Godowski P, Ashkenazi A. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* 1997; **277**: 818-821
  - 31 **Leverkus M**, Neumann M, Mengling T, Rauch CT, Brocker EB, Krammer PH, Walczak H. Regulation of tumor necrosis factor – related apoptosis-inducing ligand sensitivity in primary and transformed human keratinocytes. *Cancer Res* 2000; **60**: 553-559
  - 32 **Marsters SA**, Pitti RM, Donahue CJ, Ruppert S, Bauer KD, Ashkenazi A. Activation of apoptosis by Apo-2 ligand is independent of FADD but blocked by CrmA. *Curr Biol* 1996; **6**: 750-752
  - 33 **Wu XX**, Kakehi Y, Mizutani Y, Nishiyama H, Kamoto T, Megumi Y, Ito N, Ogawa O. Enhancement of TRAIL/Apo2L-mediated apoptosis by adriamycin through inducing DR4 and DR5 in renal cell carcinoma cells. *Int J Cancer* 2003; **104**: 409-417
  - 34 **Jo M**, Kim TH, Seol DW, Esplen JE, Dorko K, Billiar TR, Strom SC. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. *Nat Med* 2000; **6**: 564-567
  - 35 **Lawrence D**, Shahrokh Z, Marsters S, Achilles K, Shih D, Mounho B, Hillan K, Totpal K, DeForge L, Schow P, Hooley J, Sherwood S, Pai R, Leung S, Khan L, Gliniak B, Bussiere J, Smith CA, Strom SS, Kelley S, Fox JA, Thomas D, Ashkenazi A. Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. *Nature Med* 2001; **7**: 383-385
  - 36 **Qin JZ**, Chau BN, Bonish B, Nickoloff BJ. Avoiding premature apoptosis of normal epidermal cells. *Nature Med* 2001; **7**: 385-386
  - 37 **Ozoren N**, Kim K, Burns TF, Dicker DT, Moscioni AD, El-Deiry WS. The caspase-9 inhibitor Z-LEDH-FMK protects human liver cells while permitting death of cancer cells exposed to tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Res* 2000; **60**: 6259-6265
  - 38 **Nakamura TM**, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, Harley CB, Cech TR. Telomerase catalytic subunit homologs from fission yeast and human. *Science* 1997; **277**: 955-959
  - 39 **Griffith TS**, Anderson RD, Davidson BL, Williams RD, Ratliff TL. Adenoviral-mediated transfer of the TNF-related apoptosis-inducing ligand/Apo-2 ligand gene induces tumor cell apoptosis. *J Immunol* 2000; **165**: 2886-2894
  - 40 **Pan G**, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, Dixit VM. The receptor for the cytotoxic ligand TRAIL. *Science* 1997; **276**: 111-113
  - 41 **Walczak H**, Degli-Esposti MA, Johnson RS, Smolak PJ, Waugh JY, Boiani N, Timour MS, Gerhart MJ, Schooley KA, Smith CA, Goodwin RG, Rauch CT. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *EMBO J* 1997; **16**: 5386-5397

Edited by Wang XL Proofread by Zhu LH

# Preparation and characteristics of DNA-nanoparticles targeting to hepatocarcinoma cells

Qin He, Ji Liu, Xun Sun, Zhi-Rong Zhang

**Qin He, Xun Sun, Zhi-Rong Zhang**, West China School of Pharmacy, Sichuan University, Chengdu 610041, Sichuan Province, China

**Ji Liu**, West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu 610041, Sichuan Province, China

**Correspondence to:** Dr. Qin He, West China School of Pharmacy, Sichuan University, Chengdu 610041, Sichuan Province, China. qinhe317@vip.sina.com

**Telephone:** +86-28-85502532

**Received:** 2003-09-18 **Accepted:** 2003-11-19

## Abstract

**AIM:** To prepare thymidine kinase gene (TK gene) nanoparticles and to investigate the expression of TK gene.

**METHODS:** Poly(D,L-lactic-co-glycolic acid) (PLGA), a biodegradable and biocompatible polymer, was used to prepare recombinant plasmid P<sup>EGFP-AFP</sup> nanoparticles by a double-emulsion evaporation technique. Characteristics of the nanoparticles were investigated in this study, including morphology, entrapment efficiency, and tissue distribution. The expression of TK gene was also investigated by MTT assay, by which the viable cells were determined after the addition of ganciclovir (GCV). The enhanced green fluorescent protein (EGFP) expression in human hepatocellular carcinoma SMMC-7721 cells and normal parenchymal Chang liver cells were assessed by flow cytometry.

**RESULTS:** The prepared plasmid-nanoparticles had regular spherical surface and narrow particle size span with a mean diameter of 72±12 nm. The mean entrapment efficiency was 91.25%. A total of 80.14% DNA was found to be localized in the livers after 1-h injection with <sup>32</sup>P-DNA-PLGA nanoparticles in mouse caudal vein. The expression of DNA encapsulated in nanoparticles was much higher than that in naked DNA, and human hepatocellular carcinoma SMMC-7721 cells were more sensitive to GCV than human normal parenchymal Chang liver cells.

**CONCLUSION:** The enhanced transfection efficiency and stronger ability to protect plasmid DNA from being degraded by nucleases are due to nanoparticles encapsulation.

He Q, Liu J, Sun X, Zhang ZR. Preparation and characteristics of DNA-nanoparticles targeting to hepatocarcinoma cells. *World J Gastroenterol* 2004; 10(5): 660-663

<http://www.wjgnet.com/1007-9327/10/660.asp>

## INTRODUCTION

In recent years, the gene delivery system has attracted much attention<sup>[1-3]</sup>. However, safe and efficient gene delivery remains a crucial barrier to successful gene therapy. Viral and retroviral vectors have been the most efficient and commonly used delivery modalities for *in vivo* gene transfer, but viral vector may provoke mutagenesis and carcinogenesis. Repeated administration of a viral vector induces an immune response

which abolishes the transgene expression<sup>[4-7]</sup>. The non-viral delivery system has the potential to be non-immunogenic and stable *in vivo*<sup>[8-11]</sup>. Encapsulation of DNA in biodegradable polymer potentially offers a way to protect DNA from degradation and to control DNA release<sup>[12,13]</sup>, and many examples of DNA incorporated in synthetic polymers have been developed in the micron scale. Recently, some studies have shown that intracellular biodistribution of particles with diameter less than 100 nm can be achieved<sup>[14]</sup>.

Among all the present gene therapeutic protocols, combination of the administration of GCV with transfecting thymidine kinase gene of *Herpes simplex* virus (HSV-TK) into tumor cells is rather practical and potential in intra-tumoral gene therapy. The TK genes in the tumor cells can induce the metabolism of untotoxic prodrug GCV into cytotoxic parent drug, which can cause the suicide of cells. This protocol presents good potential in intra-tumoral gene therapy<sup>[15,16]</sup>. However, common TK genes (naked genes) do not have the abilities to target to specific organs and tissues, which can be harmful to the normal cells and tissues. In addition, they are easily degraded by nucleases *in vivo*.

To solve the problems mentioned above, a recombinant plasmid P<sup>EGFP-TKAFP</sup> was constructed, which can be specifically expressed in hepatocellular carcinoma cells. Furthermore, the plasmid was encapsulated in a biodegradable and biocompatible PLGA polymer to protect plasmid DNA from being digested by nucleases. The following characteristics of the nanoparticles were investigated, including *in vitro* anti-nuclease ability, tissue distribution in mice and the gene expression in hepatocellular carcinoma cells and normal parenchymal cells *in vitro*.

## MATERIALS AND METHODS

### Materials

Poly(D,L-lactic-co-glycolic acid) (PLGA; lactic-glycolic acid ratio: 75:25,  $M_n=30\,000$ , batch number: 020112) was purchased from Chengdu Institute of Organic Chemistry, Chinese Academy of Science. Recombinant plasmid P<sup>EGFP-TKAFP</sup> was a gift from Dr. Liu Ji (Sichuan University). Human hepatocellular carcinoma SMMC-7721 cells<sup>[17]</sup> and normal parenchymal Chang liver cells<sup>[18]</sup> were provided by Shanghai Institute of Cell Biology, Chinese Academy of Science. DNase I was purchased from Chengdu Huamei Biochemicals Cooperation (Sichuan Province, China). Kunming mice, weighted 18-22 g, were provided by Experimental Animal Center of Sichuan University.

### Methods

**Nanoparticles preparation** A double-emulsion evaporation technique<sup>[19]</sup> was used to prepare the nanoparticles. Briefly, plasmid DNA (200 µg) in 100 µL Tris-EDTA (TE) buffer was emulsified in 1 mL methylene chloride solution containing 100 mg of PLGA using a probe sonicator for 5 s. Polyvinyl alcohol (2 mL) was added to the primary emulsion and sonicated for another 5 s to form a double emulsion. The emulsion was added into the same concentration of polyvinyl alcohol and agitated by a magnetic stirrer for 3 h at room

temperature to remove methylene chloride.

**Particle size and morphology analysis** The PLGA nanoparticles were sized by laser diffractometry using a Malvern 2 000 laser sizer. The morphology was observed by the scanning electron microscope (JEM-100SX, Akishima, Japan). The samples were placed on to special copper grids and then stained with 20 mL/L phosphato-tungstic acid prior to visualization.

**Entrapment ratio analysis** The entrapment ratio was determined by measuring the total amount of added DNA and that of DNA being not encapsulated. In detail, colloid solution of DNA-PLGA nanoparticles was centrifuged at 45 000 *g* for 1 h. Then, the concentration of DNA in the supernatant was assessed by fluorescence spectrophotometry after stained with ethidium bromide. The exciting and emission wavelengths were 546 nm and 590 nm, respectively. The entrapment rate (ER) was calculated as follows:  $ER (\%) = \frac{DNA_{added} - DNA_{in\ the\ supernatant}}{DNA_{added}} \times 100\%$

**Protection from DNase** The PLGA nanoparticles were incubated with DNase I (0.1 unit) at 37 °C in a shaking water bath. The nanoparticles were collected by centrifugation after 4, 8, and 16 h incubation, and then chloroform was added to solubilize the nanoparticles. An equal volume of PBS solution was added, and the mixture was rotated end-over-end to facilitate the extraction of DNA from the organic phase into the aqueous phase. The samples were then centrifuged at 15 000 *g* for 15 min. The resulted supernatant was transferred to another tube and DNA was precipitated with the addition of isopropanol. Precipitate was obtained after centrifugation at 5 000 r/min for 15 min. Then, the resulted pellet was rinsed with 700 mL/L ethanol and resuspended in sterile TE buffer. The purified DNA was analyzed by gel electrophoresis.

**Tissue distribution** One hundred Kunming mice weighed 18–22 g were randomly divided into 10 test groups and 10 control groups with 5 in each group. The nanoparticles of <sup>32</sup>P-DNA-PLGA at a dose of 10 µL/g was intravenously administered to each mouse in test groups, and <sup>32</sup>P-DNA at the same dose was intravenously administered in control groups. At predetermined intervals, mice were sacrificed for blood collection. Then, heart, livers, spleen, lungs, and kidneys were removed from mice. The radioactivity of each organ was measured by a liquid scintillation analyzer.

The  $cpm_t$  was the total value of cpm in each organ ( $cpm_t$ ) at a certain time point. The ratio of  $cpm_t/cpm_i \times 100\%$  represented the relative content of DNA in viscera and blood.

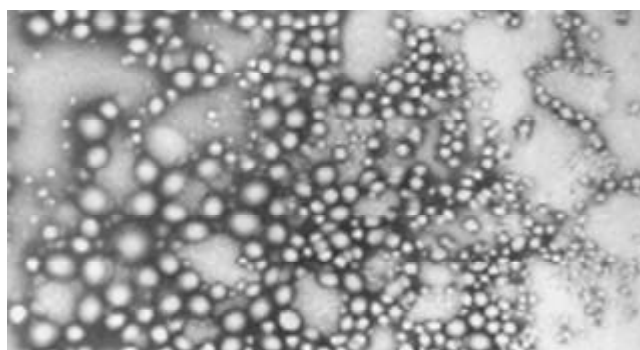
**MFI assay** Human hepatocellular carcinoma SMMC-7721 cells and normal parenchymal Chang liver cells ( $5 \times 10^5$ ) were cultured in the DMEM medium containing 100 mL/L fetal bovine serum (FBS) in 12-well plates. The cells were transfected with plasmid DNA or nanoparticles containing DNA, and maintained at 37 °C in an incubator at a 50 mL/L CO<sub>2</sub> humidified atmosphere. After incubation for 12 h, the medium was removed and replaced with fresh DMEM containing 100 mL/L FBS for further 48 h incubation. The mean fluorescence intensity (MFI) of the cells was measured by flow cytometry.

**Cytotoxicity assay** Cells were cultured in the same way as the MFI assay. After incubation for 12 h, the medium was removed, replaced with DMEM containing 100 mL/L FBS, and incubated with 0.1, 1, or 10 µg/mL GCV. The cytotoxicity of GCV was detected by MTT assay.

## RESULTS

### Size and morphology

The resulted plasmid-nanoparticles had regular spherical surface (Figure 1) and a narrow size distribution with a mean diameter of  $72 \pm 12$  nm.



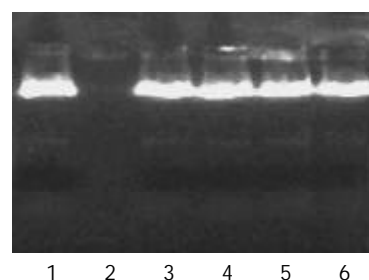
**Figure 1** Transmission electron microphotography of TK-PLGA nanoparticles.

### Entrapment efficiency

The mean entrapment efficiency was 91.25%, which was rather high in the nanoparticles preparation with PLGA as a carrier.

### Protection from DNase

Plasmid DNA encapsulated in nanoparticles remained intact in the presence of DNase I for up to 16 h incubation. On the other hand, control plasmid DNA was completely digested within 1 h incubation with the equal amount of DNase I. This result demonstrated that PLGA nanoparticles could protect encapsulated plasmid DNA from nuclease digestion (Figure 2).



**Figure 2** Agarose gel electrophoresis of DNA extracted from nanoparticles after treatment with sonication and DNase I. Lane 1 represented untreated control plasmid DNA, lane 2 represented plasmid DNA incubated with DNase I at 37 °C for 1 h, and lane 3, 4, 5, 6 indicated DNA extracted from PLGA nanoparticles incubated with DNase I at 37 °C for 0, 4, 8, or 16 h, respectively.

### Measurement of DNA in blood and viscera of mice

The tissue distribution of DNA-PLGA nanoparticles was investigated by the technique of gamma scintigraphy. The results showed that 1 h after injection with <sup>32</sup>P-DNA-PLGA nanoparticles in mouse caudal vein, the ratio of radioactivity in livers against total radioactivity was more than 80%, which was 1.5-fold of that after injection with <sup>32</sup>P-DNA alone (Table 1 and Table 2).

### Cytotoxicity assay and MFI assay

The EGFP expression in human hepatocellular carcinoma SMMC-7721 cells and normal parenchymal Chang liver cells were assessed by flow cytometry. The expression of TK gene was also investigated by MTT assay, by which the viable cells were quantitated after the addition of parent drug GCV. The results showed that the EGFP and TK expression in human hepatocellular carcinoma SMMC-7721 cells was much higher than that in human normal parenchymal Chang liver cells ( $P < 0.05$ ) (Table 3). It also showed that plasmid DNA encapsulated in nanoparticles could enhance the expression of TK or EGFP gene compared with naked plasmid DNA ( $P < 0.05$ ) (Table 4).

**Table 1** Distribution (%) of  $^{32}\text{P}$ -DNA in mice after intravenous administration of  $^{32}\text{P}$ -DNA ( $n=5$ )

Time	Heart	Liver	Spleen	Lung	Kidney	Blood
5 min	3.14±0.60	36.67±2.65	3.56±1.58	16.32±0.79	23.52±1.48	16.79±1.62
15 min	3.15±0.97	41.26±2.89	5.25±0.86	14.16±0.99	20.61±1.62	15.57±1.58
30 min	6.94±1.57	52.59±4.02	10.46±0.75	7.95±0.58	13.10±0.85	2.95±0.65
1 h	6.11±1.95	54.62±3.12	14.85±1.23	7.82±0.67	13.50±0.94	3.10±0.78
2 h	4.56±1.01	51.34±2.88	14.93±0.68	9.65±1.41	15.03±1.34	4.49±0.28
6 h	5.12±0.79	48.66±1.87	13.15±0.84	9.66±1.27	18.26±1.58	5.15±1.67
12 h	5.48±0.99	44.52±2.02	11.32±1.33	11.36±1.34	21.83±2.54	5.49±0.58
24 h	3.93±0.65	43.31±1.67	11.48±1.11	10.12±0.64	25.01±3.12	6.16±0.94
48 h	3.43±0.84	36.75±1.65	11.56±0.82	13.36±1.60	28.52±2.15	6.38±0.83
72 h	4.52±0.77	34.48±1.85	12.03±1.73	13.88±1.63	28.31±3.01	6.78±0.91

**Table 2** Distribution (%) of  $^{32}\text{P}$ -DNA in mice after intravenous administration of  $^{32}\text{P}$ -DNA-PLGA nanoparticles ( $n=5$ )

Time	Heart	Liver	Spleen	Lung	Kidney	Blood
5 min	2.22±0.51	57.32±2.36	2.96±0.69	8.29±1.33	16.58±2.02	12.63±1.24
15 min	3.52±0.64	69.69±3.32	3.19±0.58	5.77±0.55	13.87±0.99	3.96±0.36
30 min	2.38±0.67	73.37±3.62	7.55±1.03	3.64±0.41	11.28±1.28	1.78±0.65
1 h	2.08±0.54	80.14±4.56	3.37±0.67	3.08±0.62	9.34±0.68	1.99±0.54
2 h	2.59±0.28	78.45±4.02	3.73±0.76	5.35±0.58	8.36±0.94	1.47±0.23
6 h	3.18±0.60	75.03±3.69	7.38±1.03	3.74±0.39	9.61±0.96	1.06±0.24
12 h	2.21±0.51	69.34±3.96	6.17±1.24	7.88±0.64	10.17±1.04	4.22±0.32
24 h	1.94±0.32	65.59±3.25	6.46±1.04	7.95±0.57	12.10±1.23	5.96±0.59
48 h	2.21±0.29	61.18±2.69	7.01±0.48	9.27±0.71	12.87±0.86	6.46±0.86
72 h	2.51±0.37	60.56±2.98	6.45±0.69	9.98±0.65	13.54±1.11	6.96±0.75

**Table 3** Inhibition ratio of different concentrations of GCV on SMMC-7721 and Chang liver cells

GCV (μg/mL)	SMMC-7721		Chang liver	
	DNA	NP	DNA	NP
1	2.0	6.6 <sup>a</sup>	1.2	3.7
10	3.0	10.0 <sup>a</sup>	2.8	5.8

<sup>a</sup> $P<0.05$  vs Chang liver NP group; NP: nanoparticles.

**Table 4** Mean fluorescence intensity in SMMC-7721 and Chang liver cells

Cell	MFI	
	DNA	NP
SMMC-7721	0.6±0.1 <sup>a</sup>	2.1±0.3 <sup>c</sup>
Chang liver	0.6±0.2	0.7±0.2

<sup>a</sup> $P<0.05$  vs NP group; <sup>c</sup> $P<0.05$  vs Chang liver NP group; NP: nanoparticles.

## DISCUSSION

Recombinant plasmid P<sup>EGFP-AFP</sup> was constructed, which could be specifically expressed in hepatocellular carcinoma cells because of alpha-fetoprotein-albumin (AFP-alb) promoter<sup>[20-27]</sup>. Meanwhile, EGFP as the reporter gene of plasmid DNA, can be assessed by confocal laser scanning microscopy and flow cytometry<sup>[28-32]</sup>. A polymer, PLGA, was selected due to its biocompatible and biodegradable properties, which was already approved for *in vivo* applications<sup>[33-39]</sup>. Non-toxicity of the carrier may permit repeated administration of the nanoparticles to compensate for transient transgene expression.

Our results showed that plasmid DNA could be encapsulated in PLGA nanoparticles without compromising its structural and functional integrity. Additionally, PLGA nanoparticles

could protect plasmids from nuclease degradation, and therefore offer an effective approach for gene delivery *in vivo*. However, the relatively low transfection efficiency was obtained in comparison with viral vector, which still remains to be a problem.

The DNA nanoparticles probably permeate the cells through endocytotic mechanism due to their small size and negative charged surface<sup>[40]</sup>. The encapsulation of plasmid DNA in cationic liposomes offers another choice to be protected from DNases. However, cationic liposomes may be toxic to cells due to an excess of positive charge<sup>[41,42]</sup>, and can be easily influenced by the substances in plasma. In recent years, nanoparticles attract more and more attention because of many advantages, including high stability at room temperature, favorable safety, the ability to deliver plasmid DNA at a controllable rate, and easy adaptability. Unlike most viral vector, there is no limit on the size of plasmids encapsulated into the nanoparticles<sup>[43,44]</sup>.

## REFERENCES

- 1 **Wiethoff CM**, Middaugh CR. Barriers to nonviral gene delivery. *J Pharm Sci* 2003; **92**: 203-217
- 2 **Guo SY**, Gu QL, Zhu ZG, Hong HQ, Lin YZ. TK gene combined with mIL-2 and mGM-CSF genes in treatment of gastric cancer. *World J Gastroenterol* 2003; **9**: 233-237
- 3 **Mhashilkar A**, Chada S, Roth JA, Ramesh R. Gene therapy. Therapeutic approaches and implications. *Biotechnol Adv* 2001; **19**: 279-297
- 4 **Liu F**, Huang L. Development of non-viral vectors for systemic gene delivery. *J Control Release* 2002; **78**: 259-266
- 5 **Corsi K**, Chellat F, Yahia L, Fernandes JC. Mesenchymal stem cells, MG63 and HEK293 transfection using chitosan-DNA nanoparticles. *Biomaterials* 2003; **24**: 1255-1264
- 6 **Brown MD**, Schatzlein AG, Uchegbu IF. Gene delivery with synthetic (non viral) carriers. *Int J Pharm* 2001; **229**: 1-21
- 7 **Kirchheis R**, Wightman L, Wagner E. Design and gene delivery activity of modified polyethylenimines. *Adv Drug Deliv Rev* 2001; **53**: 341-358

- 8 **Hashida M**, Nishikawa M, Yamashita F, Takakura Y. Cell-specific delivery of genes with glycosylated carriers. *Adv Drug Deliv Rev* 2001; **52**: 187-196
- 9 **Kamiya H**, Tsuchiya H, Yamazaki J, Harashima H. Intracellular trafficking and transgene expression of viral and non-viral gene vectors. *Adv Drug Deliv Rev* 2001; **52**: 153-164
- 10 **Zhdanov RI**, Podobed OV, Vlassov VV. Cationic lipid-DNA complexes-lipoplexes-for gene transfer and therapy. *Bioelectrochemistry* 2002; **58**: 53-64
- 11 **Oku N**, Yamazaki Y, Matsuura M, Sugiyama M, Hasegawa M, Nango M. A novel non-viral gene transfer system, polycation liposomes. *Adv Drug Deliv Rev* 2001; **52**: 209-218
- 12 **Crommelin DJ**, Storm G, Jiskoot W, Stenekes R, Mastrobattista E, Hennink W. Nanotechnological approaches for the delivery of macromolecules. *J Control Release* 2003; **87**: 81-88
- 13 **Guang Liu W**, De Yao K. Chitosan and its derivatives- a promising non-viral vector for gene transfection. *J Control Release* 2002; **83**: 1-11
- 14 **Hirosue S**, Muller BG, Mulligan RC, Langer R. Plasmid DNA encapsulation and release from solvent diffusion nanospheres. *J Control Release* 2001; **70**: 231-242
- 15 **Singh S**, Cunningham C, Buchanan A, Jolly D, Nemunaitis J. Toxicity assessment of intratumoral injection of the herpes simplex type I thymidine kinase gene delivered by retrovirus in patients with refractory cancer. *Mol Ther* 2001; **4**: 157-160
- 16 **Vlachaki MT**, Chhikara M, Aguilar L, Zhu X, Chiu KJ, Woo S, Teh BS, Thompson TC, Butler EB, Aguilar-Cordova E. Enhanced therapeutic effect of multiple injections of HSV-TK +GCV gene therapy in combination with ionizing radiation in a mouse mammary tumor model. *Int J Radiat Oncol Biol Phys* 2001; **51**: 1008-1017
- 17 **Dong RC**, Zhou RH, Lu FD, Tao WZ. Primary study on the establishment of Human hepatocellular carcinoma cell line SMMC-7721 and its biological characteristics. *Dier Junyi Daxue Xuebao* 1980; **1**: 5-9
- 18 **Chang RS**. Continuous subcultivation of epithelial-like cells from normal human tissues. *Proc Soc Exp Biol Med* 1954; **87**: 440-443
- 19 **Prabha S**, Zhou WZ, Panyam J, Labhasetwar V. Size-dependency of nanoparticle-mediated gene transfection: studies with fractionated nanoparticles. *Int J Pharm* 2002; **244**: 105-115
- 20 **Sa Cunha A**, Bonte E, Dubois S, Chretien Y, Eraiser T, Degott C, Brechot C, Tran PL. Inhibition of rat hepatocellular carcinoma tumor growth after multiple infusions of recombinant Ad.AFPtk followed by ganciclovir treatment. *J Hepatol* 2002; **37**: 222-230
- 21 **Li MS**, Li PF, He SP, Du GG, Li G. The promoting molecular mechanism of alpha-fetoprotein on the growth of human hepatoma Bel 7402 cell line. *World J Gastroenterol* 2002; **8**: 469-475
- 22 **Cao G**, Kuriyama S, Gao J, Nakatani T, Chen Q, Yoshiji H, Zhao L, Kojima H, Dong Y, Fukui H, Hou J. Gene therapy for hepatocellular carcinoma based on tumour-selective suicide gene expression using the alpha-fetoprotein (AFP) enhancer and a house-keeping gene promoter. *Eur J Cancer* 2001; **37**: 140-147
- 23 **Kim J**, Lee B, Kim JS, Yun CO, Kim JH, Lee YJ, Joo CH, Lee H. Antitumoral effects of recombinant adenovirus YKL-1001, conditionally replicating in alpha-fetoprotein-producing human liver cancer cells. *Cancer Lett* 2002; **180**: 23-32
- 24 **Ishikawa H**, Nakata K, Mawatari F, Ueki T, Tsuruta S, Ido A, Nakao K, Kato Y, Ishii N, Eguchi K. Retrovirus-mediated gene therapy for hepatocellular carcinoma with reversely oriented therapeutic gene expression regulated by alpha-fetoprotein enhancer/promoter. *Biochem Biophys Res Commun* 2001; **287**: 1034-1040
- 25 **Takahashi M**, Sato T, Sagawa T, Lu Y, Sato Y, Lyama S, Yamada Y, Fukaura J, Takahashi S, Miyanishi K, Yamashita T, Sasaki K, Kogawa K, Hamada H, Kato J, Niitsu Y. E1B-55K-deleted adenovirus expressing E1A-13S by AFP-enhancer/promoter is capable of highly specific replication in AFP-producing hepatocellular carcinoma and eradication of established tumor. *Mol Ther* 2002; **5**(5 Pt 1): 627-634
- 26 **Ye X**, Liang M, Meng X, Ren X, Chen H, Li ZY, Ni S, Lieber A, Hu F. Insulation from viral transcriptional regulatory elements enables improvement to hepatoma-specific gene expression from adenovirus vectors. *Biochem Biophys Res Commun* 2003; **307**: 759-764
- 27 **Lu SY**, Sui YF, Li ZS, Pan CE, Ye J, Wang WY. Construction of a regulable gene therapy vector targeting for hepatocellular carcinoma. *World J Gastroenterol* 2003; **9**: 688-691
- 28 **Kantakamalakul W**, Jaroenpool J, Pattanapanyasat K. A novel enhanced green fluorescent protein (EGFP)-K562 flow cytometric method for measuring natural killer (NK) cell cytotoxic activity. *J Immunol Methods* 2003; **272**: 189-197
- 29 **Bi JX**, Wirth M, Beer C, Kim EJ, Gu MB, Zeng AP. Dynamic characterization of recombinant Chinese hamster ovary cells containing an inducible c-fos promoter GFP expression system as a biomarker. *J Biotechnol* 2002; **93**: 231-242
- 30 **Henry SC**, Schmader K, Brown TT, Miller SE, Howell DN, Daley GG, Hamilton JD. Enhanced green fluorescent protein as a marker for localizing murine cytomegalovirus in acute and latent infection. *J Virol Methods* 2000; **89**: 61-73
- 31 **Jakobs S**, Subramaniam V, Schonle A, Jovin TM, Hell SW. EFGP and DsRed expressing cultures of *Escherichia coli* imaged by confocal, two-photon and fluorescence lifetime microscopy. *FEBS Lett* 2000; **479**: 131-135
- 32 **Hanson P**, Mathews V, Marrus SH, Graubert TA. Enhanced green fluorescent protein targeted to the Sca-1 (Ly-6A) locus in transgenic mice results in efficient marking of hematopoietic stem cells *in vivo*. *Exp Hematol* 2003; **31**: 159-167
- 33 **Yamaguchi Y**, Takenaga M, Kitagawa A, Ogawa Y, Mizushima Y, Igarashi R. Insulin-loaded biodegradable PLGA microcapsules: initial burst release controlled by hydrophilic additives. *J Control Release* 2002; **81**: 235-249
- 34 **Perez-Rodriguez C**, Montano N, Gonzalez K, Griebenow K. Stabilization of  $\alpha$ -chymotrypsin at the CH<sub>2</sub>Cl<sub>2</sub>/water interface and upon water-in-oil-in-water encapsulation in PLGA microspheres. *J Control Release* 2003; **89**: 71-85
- 35 **Dorta MJ**, Santovena A, Llabres M, Farina JB. Potential applications of PLGA film-implants in modulating *in vitro* drugs release. *Int J Pharm* 2002; **248**: 149-156
- 36 **Lu L**, Yaszemski MJ, Mikos AG. Retinal pigment epithelium engineering using synthetic biodegradable polymers. *Biomaterials* 2001; **22**: 3345-3355
- 37 **Jain RA**, Rhodes CT, Railkar AM, Malick AW, Shah NH. Controlled release of drugs from injectable *in situ* formed biodegradable PLGA microspheres: effect of various formulation variables. *Eur J Pharm Biopharm* 2000; **50**: 257-262
- 38 **Panyam J**, Labhasetwar V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. *Adv Drug Deliv Rev* 2003; **55**: 329-347
- 39 **Vila A**, Sanchez A, Tobio M, Calvo P, Alonso MJ. Design of biodegradable particles for protein delivery. *J Control Release* 2002; **78**: 15-24
- 40 **Garcia-Chaumont C**, Seksek O, Grzybowska J, Borowski E, Bolard J. Delivery systems for antisense oligonucleotides. *Pharmacol Ther* 2000; **87**: 255-277
- 41 **Han SO**, Mahato RI, Kim SW. Water-soluble lipopolymer for gene delivery. *Bioconjug Chem* 2001; **12**: 337-345
- 42 **Pouton CW**, Seymour LW. Key issues in non-viral gene delivery. *Adv Drug Deliv Rev* 2001; **46**: 187-203
- 43 **del Barrio GG**, Novo FJ, Irache JM. Loading of plasmid DNA into PLGA microparticles using TROMS (Total Recirculation One-Machine System): evaluation of its integrity and controlled release properties. *J Control Release* 2003; **86**: 123-130
- 44 **Davis ME**. Non-viral gene delivery systems. *Curr Opin Biotechnol* 2002; **13**: 128-131

# Inhibitor RNA blocks the protein translation mediated by hepatitis C virus internal ribosome entry site *in vivo*

Xue-Song Liang, Jian-Qi Lian, Yong-Xing Zhou, Mo-Bin Wan

**Xue-Song Liang, Mo-Bin Wan**, Department of Infectious Diseases, Changhai Hospital, Second Military Medical University, Shanghai, China

**Jian-Qi Lian, Yong-Xing Zhou**, Department of Infectious Diseases, Tangdu Hospital, Fourth Military Medical University, Xi'an 710038, Shaanxi Province, China

**Supported by** the National Natural Science Foundation of China, No. 30000147

**Co-correspondents:** Xue-Song Liang and Jian-Qi Lian

**Correspondence to:** Dr. Xue-Song Liang, Department of Infectious Diseases, Changhai Hospital, Second Military Medical University, Shanghai, China. liangxuesong2000@163.com

**Telephone:** +86-21-25072109

**Received:** 2003-08-08 **Accepted:** 2003-10-07

## Abstract

**AIM:** To investigate the inhibitory effect of hepatitis C virus internal ribosome entry site (HCV IRES) specific inhibitor RNA (IRNA) on gene expression mediated by HCV IRES *in vivo*.

**METHODS:** By using G418 screening system, hepatoma cells constitutively expressing IRNA or mutant IRNA (mIRNA) were established and characterized, and HCV replicons containing the 5' untranslated region (5' UTR) were constructed by using the same method. Cotransfection of pCMVNCRLuc containing HCV 5' UTR-luc fusion genes and eukaryotic vector of IRNA into human hepatic carcinoma cells (HepG2) was performed and the eukaryotic expression plasmid of IRNA was transfected transiently into HCV replicons. pCMVNCRLuc or pCDNA-luc was cotransfected with pSV40-β Gal into IRNA expressing hepatoma cells by using lipofectamine 2000 *in vitro*. Then the reporting gene expression level was examined at 48 h after transfection by using a luminometer and the expressing level of HCV C antigen was analysed with a confocal microscope.

**RESULTS:** Transient expression of IRES specific IRNA could significantly inhibit the expression of reporter gene and viral antigen mediated by HCV IRES by 50% to 90% *in vivo*, but mIRNA lost its inhibitory activity completely. The luciferase gene expression mediated by HCV IRES was blocked in the HHCC constitutively expressing IRNA. At 48 h after transfection, the expression level of reporter gene decreased by 20%, but cap-dependent luciferase gene expression was not affected. IRNA could inhibit the HCV replicon expression 24 h after transfection and the highest inhibitory activity was 80% by 72 h, and the inhibitory activity was not increased until 7d after transfection.

**CONCLUSION:** IRNA can inhibit HCV IRES mediated gene expression *in vivo*.

Liang XS, Lian JQ, Zhou YX, Wan MB. Inhibitor RNA blocks the protein translation mediated by hepatitis C virus internal ribosome entry site *in vivo*. *World J Gastroenterol* 2004; 10 (5): 664-667

<http://www.wjgnet.com/1007-9327/10/664.asp>

## INTRODUCTION

Hepatitis C virus (HCV) is the primary causative agent of parenterally transmitted non-A, non-B hepatitis and affects a significant part of the world population. HCV infection frequency leads to chronic hepatitis, cirrhosis of the liver, and hepatocellular carcinoma. The genome of HCV is a single-stranded, plus-polarity RNA. The 5' untranslated region (UTR) of HCV RNA is approximately 340 nt long, and contains multiple AUG codons. The 5' UTR is highly conserved among different strains of HCV. Nucleotides 40 to 370 of the 5' UTR of HCV have been shown to contain an internal ribosome entry site (IRES)<sup>[1-3]</sup>. The presence and stability of IRES play an important role in virus life cycle, so the region has become the target of antiviral gene therapy<sup>[4-16]</sup>. Coward and Dasgupta found that gene expression mediated by polio virus (PV) IRES was inhibited by one 60 nt long RNA which is called inhibitor RNA (IRNA). Because HCV and PV IRES elements bound to similar polypeptides<sup>[16-20]</sup>, it was reasoned that IRNA might also interfere with HCV IRES-mediated translation. Using transient transfection of hepatoma cells and a hepatoma cell line constitutively expressing IRNA, we demonstrated specific inhibition of HCV IRES-mediated translation by IRNA *in vivo*.

## MATERIALS AND METHODS

### Materials

Vectors pCRz-IRNA and pCRz-mIRNA were constructed by our laboratory, which introduced the sequences of 5' and 3' *cis*-self cleavage ribozyme into both sides of IRNA or mIRNA sequence<sup>[21]</sup>. pCMVNCRLuc contain full sequence of HCV 5' UTR and 66 nt core gene, and was fused with luciferase gene (generous gift of professor Alt). pCHCVcluc was constructed by our laboratory containing full sequence of HCV 5' UTR and partial sequence of core region, and could express in cells stably.

### Methods

**Cell culture** Human hepatocarcinoma cell (HHCC) HepG2 was grown in RPMI1640 medium supplemented with 100 mL/L newborn calf serum.

**Plasmid construction** By using subcloning methods, IRNA and mIRNA sequence were cloned into the pcDNA3 vector, yielding pCRz-IRNA and pCRz-mIRNA which introduced the ribozyme sequence over both sides of IRNA and mIRNA to generate the correct side of IRNA and mIRNA<sup>[16]</sup>. In brief, by using PCR methods the sequences of target RNA were generated from pGRz-IRNA or pGRz-mIRNA which was constructed by our laboratory. Then the PCR product was cloned into the *Bam*HI-*Apa*I sites of the pcDNA3 vector.

**Establishment of stable hepatoma cell line expressing IRNA or cloning HCV replicon** Plasmids pCRz-IRNA, pCRz-mIRNA, pcDNA3 and pCHCVcluc were transfected into HHCC respectively by using Lipofectamine 2000 reagent (GIBCO) and screened for neomycin resistance with 300 µg/mL of geneticin (G418) (Invitrogen) per milliliter for 4 weeks. The antibiotic-resistant cell clones were harvested and further screened by dilution titer.



**Detection of IRNA in cell lines** IRNA or miRNA expression in the cells was measured by isolating total RNA from these cells and IRNA or miRNA were detected by reverse transcriptase (RT)-mediated PCR (RT-PCR) by using IRNA or miRNA specific oligonucleotide primers. One to 2  $\mu$ g of total RNA isolated from the IRNA or miRNA expressing cells, and 2  $\mu$ g total RNA from HHCC control cells were reversely transcribed by murine leukemia virus RT using random hexamer primers in 20  $\mu$ L reaction mixture according to the TaKaRa RNA PCR kit protocol. Twenty pmol of each primer (corresponding to 5' nt 1 to 20 and 3' nt 1 to 20 of the IRNA or miRNA sequence) was used to amplify the 60-nt fragment in 100  $\mu$ L PCR reaction. The cycling parameters were as follows: denaturation at, 95 °C for 1 min, annealing at, 65 °C for 1 min, extension at, 72 °C for 1 min, a total of 50 cycles, then total extension at, 72 °C for 10 min. Twenty microliters of each reaction product were loaded onto 20 g/L gel and visualized by ethidium bromide staining.

**Detection of HCV core protein expression in HHCC** HCV core protein was detected by using indirect immune fluorescence method. HCV replicon cells were plated on a cover glass and fixed with pure ethanol for 10 min. Monoclonal antibody of HCV core protein was properly diluted (1:100) and covered on the glass with HCV replicon cells for 1 h at 37 °C, and then the glass was washed 3 times with PBS (10 min each). Then FITC labeled second antibody was covered on the glass at 37 °C for 1 h and the glass was washed 3 times again with PBS. At last the cells were examined by using fluorescence microscopy or laser confocal microscopy.

**DNA transfection** For each transfection assay,  $1 \times 10^6$  HHCC cells in 30-mm-diameter plates were transfected with 15  $\mu$ L of lipofectin (GIBCO) and 2 to 5  $\mu$ g of plasmid DNA. At 16 h post transfection, cell lysates were prepared according to the luciferase assay kit protocol (Promega) and assayed for both  $\beta$ -galactosidase ( $\beta$ -Gal) and luciferase expression.

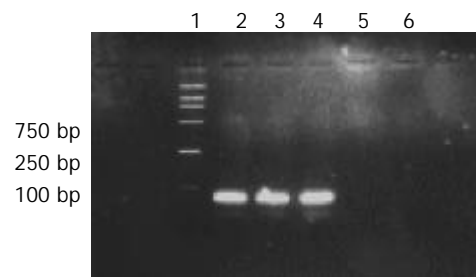
## RESULTS

### Inhibitor effect of IRNA transient expression on HCV IRES-mediated translation

To test the possibility that IRNA interfered with HCV IRES-mediated translation, human hepatocellular carcinoma cells (HepG2) were transiently cotransfected with three plasmids: a reporter gene expressing luciferase programmed by the HCV IRES element (pCMVNCRLuc), pSV- $\beta$ -galactosidase to measure transfection efficiency, and the plasmid expressing IRNA (pCRz-IRNA). All transfections were done in triplicate and contained equal amounts of the luciferase reporter and  $\beta$ -Gal plasmid. Increasing concentrations of plasmid pCRz-IRNA were used in various reactions, and the total amount of DNA in each reaction was kept constant by addition of an appropriate amount of a nonspecific DNA (pcDNA3). Following transfection, luciferase activity was measured in cell extracts at 48 h. At the lowest concentration of the IRNA plasmid, inhibition of luciferase activity from plasmid pCMVNCRLuc was approximately 50% compared to the control. However, at the highest concentration, 92% of luciferase activity was inhibited. Translation of luciferase from

a control plasmid (pCDNA-luc) without HCV IRES was not significantly inhibited by IRNA, ( $P > 0.05$ , Table 1).

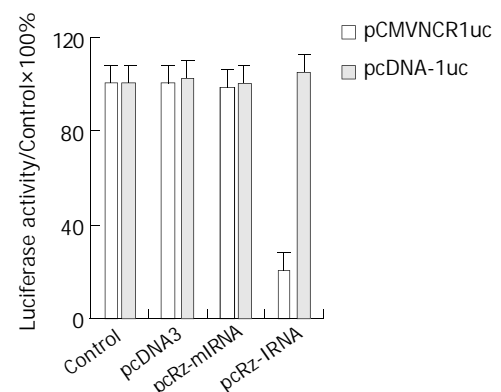
In order to determine the inhibitor effect of IRNA on HCV IRES-mediated translation further, the HHCCs were transfected with pCMVNCRLuc or cotransfected with pCRz-IRNA and pCMVNCRLuc. Following transfection, the HCV core protein programmed by HCV IRES was detected by using laser confocal microscopy. HCV core protein could express efficiently in the HHCC cells as shown in Figure 1. But IRNA plasmid cotransfection could inhibit HCV core protein expression. The pels density of HCV core protein was different between the two groups ( $58.05 \pm 42.24$  vs  $15.56 \pm 8.54$ ). The inhibitory rate was plotted by  $1 - \text{pels density of IRNA transfection group} / \text{pels density of control} \times 100\%$ .



**Figure 1** Stable expression of IRNA and miRNA in HHCC cells. 1: DL2000 DNA marker; 2, 3: IRNA RT-PCR; 4: miRNA RT-PCR; 5: IRNA PCR.

### Construction of hepatoma cell line expressing IRNA or miRNA

To determine the long-term effect of RNA expression in HepG2, the cell line constitutionally expressing IRNA was generated by using a pcDNA-based vector as described in Material and Methods. In order to obtain both the correct and stable sites of the expressed IRNA, ribozyme sequences were introduced into both sides of IRNA and miRNA. IRNA or miRNA was examined by RT-PCR using appropriate primers. IRNA and miRNA were expressed stably in the stable cell lines as shown in Figure 2.



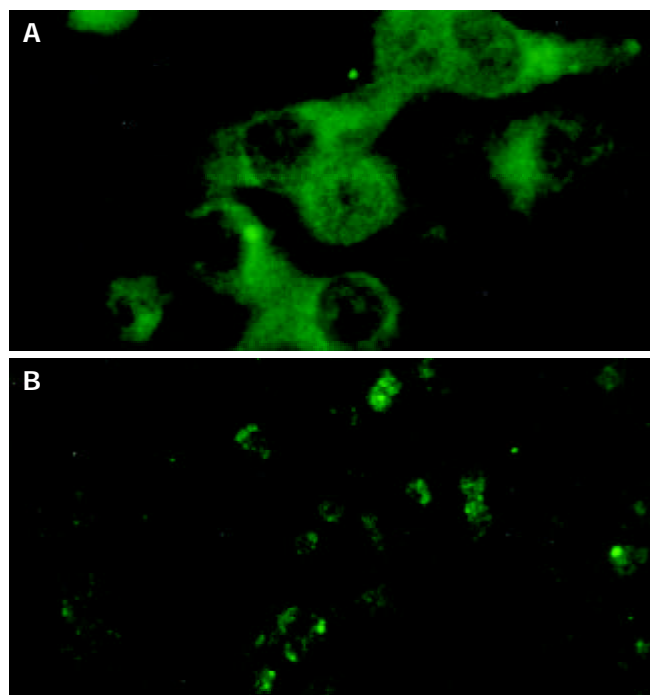
**Figure 2** Inhibitory effect of IRNA on luciferase expression mediated by different mechanism.

**Table 1** Inhibitory effect of IRNA on HCV IRES mediated gene translation

Sample pCRz-IRNA $\mu$ g	Activity of luciferase programmed by cap-dependent mechanism (IU/U)	Activity of luciferase programmed by HCV IRES (IU/U)	Inhibitory rate (%)	
			pcDNA-luc	pCMVNCRLuc
0	55.5 $\pm$ 3.11	52.49 $\pm$ 2.31		
2	60.1 $\pm$ 2.31	27.0 $\pm$ 0.740	0	50
4	58.3 $\pm$ 1.89	14.7 $\pm$ 0.380	0	72
6	52.4 $\pm$ 2.12	4.03 $\pm$ 0.120	4	92

### HCV IRES-mediated gene expression in IRNA expressing HepG2 cells

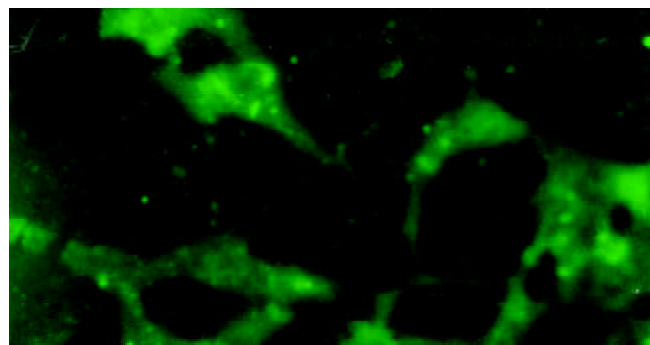
IRNA or miRNA expressing HepG2 cells and empty vector pcDNA3 expressing cells or control cells were cotransfected with pCMVNCRLuc and transfection efficiency control plasmid pSV- $\beta$ -Gal. At 48 h post-transfection cell extracts were used to measure the activities of both luciferase and  $\beta$ -galactosidase. The result was plotted as percent of control after normalized for  $\beta$ -Gal activity and protein concentration. The pcRz-IRNA cells showed approximately 80% inhibition of luciferase activity compared to the control. Both pcRz-miRNA cells and pcDNA3 cells showed less than 5% inhibition activity. No significant inhibition of cap-dependent translation from the pCDNA-luc construct was observed in cell lines expressing IRNA, ( $P=0.05$ , Figure 3).



**Figure 3** Inhibitory effect of IRNA on HCV Core expression mediated by HCV IRES(A: 40 $\times$ 10 vs B: 25 $\times$ 10). A: HCV Core protein of pCMVNCRLuc transfection group, B: HCV Core protein of pcRz-IRNA and pCMVNCRLuc cotransfection group.

### Construction of HCV replicon containing HCV IRES

The results demonstrated that HCV core programmed by HCV IRES was positive in about 90% of HHCC cells (Figure 4).

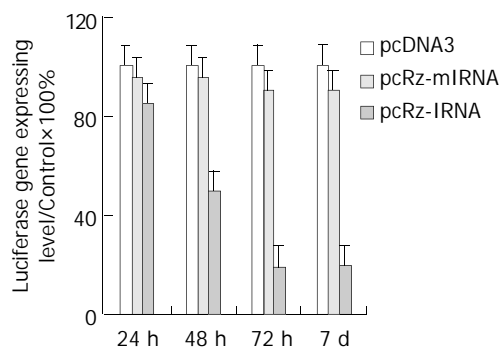


**Figure 4** Detection of HCV core protein in HHCC lines stably expressing pcHCV Cluc (25 $\times$ 10).

### Interference of IRNA with HCV replicon translation

To confirm the result obtained in IRNA expressing cells, HCV

replicon containing HCV IRES was transfected with IRNA expressing plasmid, and luciferase activity was determined at different time following transfection. The result was that at 24 h HCV IRES-mediated luciferase gene translation decreases by 15% compared to the control HHCC cells, and along with time extending, the inhibitory effect of IRNA on HCV IRES-mediated luciferase gene translation increased and reached 80% at 72 h. On d 7, the inhibitory effect was still 80%. But miRNA and nonspecific short RNA did not show any inhibitory effect on HCV IRES-mediated gene translation (Figure 5).



**Figure 5** Interference of IRNA with HCV replicon translation.

### DISCUSSION

IRES-dependent protein translation mechanism was first discovered in picornaviruses, including PV, rhinovirus and hepatitis A virus, as well as certain flaviviruses, such as hepatitis C virus<sup>[23-28]</sup>. Although there is very little sequence homology between these different IRES elements, structural similarity does appear to exist. In fact, in order to keep the activity of IRES, it was more important to maintain the secondary structure than to maintain the integrality of certain genome sequences<sup>[1-3,7,8]</sup>. IRES is the key structure for some viral RNA replication, so it has become the target for antiviral infection. We have constructed the self-cleavage plasmid of IRNA, and affirmed that IRNA can inhibit IRES-dependent protein translation *in vitro*<sup>[22]</sup>. In order to further confirm the effect of long-term expressing IRNA on cellular protein and viral protein translation, we established a HHCC line stably expressing IRNA, and confirmed that long-term expressing IRNA could significantly inhibit IRES mediated protein translation compared to the control cells and miRNA expressing cells. Das *et al* prepared the human hepatoma (Huh-7) cell lines expressing IRNA by using the similar methods. They found that HCV IRES-mediated cap-independent translation was markedly inhibited in cells constitutively expressing IRNA compared to control hepatoma cells<sup>[29]</sup>.

Alt *et al* designed the vector pCMVNCRLuc fusing the luciferase gene to HCV core gene 66 nt site, and the gene expression was mediated by HCV 5' UTR, so we could determine the inhibitor effect of new strategies on HCV 5' UTR by examining the activity of luciferase. In this study, three plasmids pCMVNCRLuc, pcRz-IRNA expressing IRNA and transfection efficiency control plasmid pSV- $\beta$ Gal were cotransfected into HHCC cells and luciferase activity (light units) was expressed as percentage of the control after normalized for  $\beta$  gal activity. When the effect of transfection efficiency and transient expression efficiency were excluded, the results of this study showed that IRNA could specifically inhibit HCV IRES mediated gene expression *in vivo*. The results of our study suggested that HCV 5' UTR-mediated translation was specifically inhibited by IRNA transient expression in hepatoma cells (50% to 92%), whereas cap-dependent translation of luciferase from the control plasmid

lack of HCV IRES element was not significantly affected by IRNA. To confirm the result obtained by using transient transfection, the vector containing HCV IRES was transfected into human hepatoma cells expressing IRNA constitutively and the results demonstrated that stably expressing IRNA could inhibit HCV IRES-mediated translation. By using a bicistronic construct containing CAT and luciferase genes flanked by the HCV 5' UTR Das *et al* found that IRNA could significantly inhibit HCV IRES-mediated gene expression *in vitro*. Further, they studied the IRNA effect *in vivo* and obtained the similar result to our study<sup>[29,30]</sup>.

In order to determine the IRNA inhibitor effect *in vivo* further, we used the HCV replicon containing the full length of HCV 5' UTR to investigate the IRNA activity; the results demonstrated that IRNA could inhibit HCV 5' UTR mediated gene expression *in vivo*, but IRNA could not completely block HCV 5' UTR mediated gene expression.

To rule out the nonspecific effect of nonspecific short RNA regiment on HCV IRES-mediated gene expression, plasmids pCDNA3 and pCMVNCRLuc were cotransfected into human hepatoma cells and the results showed that nonspecific RNA regiment didn't have the inhibitor effect on HCV IRES-mediated translation.

In summary, IRNA can significantly inhibit HCV IRES-mediated translation.

## REFERENCES

- Friebe P**, Lohmann V, Krieger N, Bartenschlager R. Sequences in the 5' nontranslated region of hepatitis C virus required for RNA replication. *J Virol* 2001; **75**: 12047-12057
- Jubin R**. Hepatitis C IRES: translating translation into a therapeutic target. *Curr Opin Mol Ther* 2001; **3**: 278-287
- Reusken CB**, Dalebout TJ, Eerligh P, Bredenbeek PJ, Spaan WJ. Analysis of hepatitis C virus/classical swine fever virus chimeric 5' NTRs: sequences within the hepatitis C virus IRES are required for viral RNA replication. *J Gen Virol* 2003; **84**(Pt 7): 1761-1769
- Klinck R**, Westhof E, Walker S, Afshar M, Collier A, Aboul-Ela F. A potential RNA drug target in the hepatitis C virus internal ribosomal entry site. *RNA* 2000; **6**: 1423-1431
- Wang W**, Preville P, Morin N, Mounir S, Cai W, Siddiqui MA. Hepatitis C viral IRES inhibition by phenazine and phenazine-like molecules. *Bioorg Med Chem Lett* 2000; **10**: 1151-1154
- Shimazaki T**, Honda M, Kaneko S, Kobayashi K. Inhibition of internal ribosomal entry site-directed translation of HCV by recombinant IFN- $\alpha$  correlates with a reduced La protein. *Hepatology* 2002; **35**: 199-208
- Gallego J**, Varani G. The hepatitis C virus internal ribosome-entry site: a new target for antiviral research. *Biochem Soc Trans* 2002; **30**: 140-145
- Vyas J**, Elia A, Clemens MJ. Inhibition of the protein kinase PKR by the internal ribosome entry site of hepatitis C virus genomic RNA. *RNA* 2003; **9**: 858-870
- Kikuchi K**, Umehara T, Fukuda K, Hwang J, Kuno A, Hasegawa T, Nishikawa S. RNA aptamers targeted to domain II of hepatitis C virus IRES that bind to its apical loop region. *J Biochem* 2003; **133**: 263-270
- Liang XS**, Lian JQ, Zhou YX, Nie QH, Hao CQ. A small yeast RNA inhibits HCV IRES mediated translation and inhibits replication of poliovirus *in vivo*. *World J Gastroenterol* 2003; **9**: 1008-1013
- He Y**, Yan W, Coito C, Li Y, Gale M Jr, Katze MG. The regulation of hepatitis C virus (HCV) internal ribosome-entry site-mediated translation by HCV replicons and nonstructural proteins. *J Gen Virol* 2003; **84**(Pt 3): 535-543
- Otto GA**, Lukavsky PJ, Lancaster AM, Sarnow P, Puglisi JD. Ribosomal proteins mediate the hepatitis C virus IRES-HeLa 40S interaction. *RNA* 2002; **8**: 913-923
- Das S**, Ott M, Yamane A, Venkatesan A, Gupta S, Dasgupta A. Inhibition of internal entry site (IRES)-mediated translation by a small yeast RNA: a novel strategy to block hepatitis C virus protein synthesis. *Front Biosci* 1998; **3**: D1241-1252
- Das S**, Coward P, Dasgupta A. A small yeast RNA selectively inhibits internal initiation of translation programmed by poliovirus RNA: specific interaction with cellular proteins that bind to the viral 5'-untranslated region. *J Virol* 1994; **68**: 7200-7211
- Kikuchi K**, Umehara T, Fukuda K, Hwang J, Kuno A, Hasegawa T, Nishikawa S. Structure-inhibition analysis of RNA aptamers that bind to HCV IRES. *Nucleic Acids Res Suppl* 2003; **3**: 291-292
- Liang X**, Zhou Y, Lian J, Nie Q, Jia Z. Effect of inhibitor RNA on intracellular inhibition of viral gene expression in 5'-noncoding region of hepatitis C virus. *Zhonghua Neike Zazhi* 2002; **41**: 660-662
- Das S**, Kenan DJ, Bocskai D, Keene JD, Dasgupta A. Sequences within a small yeast RNA required for inhibition of internal initiation of translation: interaction with La and other cellular proteins influences its inhibitory activity. *J Virol* 1996; **70**: 1624-1632
- Isoyama T**, Kamoshita N, Yasui K, Iwai A, Shiroki K, Toyoda H, Yamada A, Takasaki Y, Nomoto A. Lower concentration of La protein required for internal ribosome entry on hepatitis C virus RNA than on poliovirus RNA. *J Gen Virol* 1999; **80**(Pt 9): 2319-2327
- Gamamik AV**, Andino R. Interactions of viral protein 3CD and poly(rC) binding protein with the 5' untranslated region of the poliovirus genome. *J Virol* 2000; **74**: 2219-2226
- Ray PS**, Das S. La autoantigen is required for the internal ribosome entry site-mediated translation of Coxsackievirus B3 RNA. *Nucleic Acids Res* 2002; **30**: 4500-4508
- Ali N**, Pruijn GJ, Kenan DJ, Keene JD, Siddiqui A. Human La antigen is required for the hepatitis C virus internal ribosome entry site-mediated translation. *J Biol Chem* 2000; **275**: 27531-27540
- Liang XS**, Zhou YX, Lian JQ, Hao CQ, Wang LX. Structure modeling and construction recombinant plasmid of HCV IRES specific inhibitor RNA (IRNA). *J Med Post* 2002; **15**: 189-192
- Jin J**, Yang JY, Liu J, Kong YY, Wang Y, Li GD. DNA immunization with fusion genes encoding different regions of hepatitis C virus E2 fused to the gene for hepatitis B surface antigen elicits immune responses to both HCV and HBV. *World J Gastroenterol* 2002; **8**: 505-510
- Woitak RP**, Petersen U, Moshage D, Brackmann HH, Matz B, Sauerbruch T, Spengler U. HCV-specific cytokine induction in monocytes of patients with different outcomes of hepatitis C. *World J Gastroenterol* 2002; **8**: 562-566
- Yu YC**, Mao Q, Gu CH, Li QF, Wang YM. Activity of HDV ribozymes to trans-cleave HCV RNA. *World J Gastroenterol* 2002; **8**: 694-698
- Tang BZ**, Zhuang L, You J, Zhang HB, Zhang L. Seven-years follow-up on trial of Interferon  $\alpha$  in patients with HCV RNA positive chronic hepatitis C. *World J Gastroenterol* 2000; **6**: 68
- Li LF**, Zhou Y, Xia S, Zhao LL, Wang ZX, Wang CQ. The epidemiologic feature of HCV prevalence in Fujian. *World J Gastroenterol* 2000; **6**: 80
- Kato J**, Kato N, Moriyama M, Goto T, Taniguchi H, Shiratori Y, Omata M. Interferons specifically suppress the translation from the internal ribosome entry site of hepatitis C virus through a double-stranded RNA-activated protein kinase-independent pathway. *J Infect Dis* 2002; **186**: 155-163
- Das S**, Kenan DJ, Bocskai D, Keene JD, Dasgupta A. Sequences within a small yeast RNA required for inhibition of initiation of translation: interaction with La and other cellular proteins influences its inhibitory activity. *J Virol* 1996; **70**: 1624-1632
- Venkatesan A**, Das S, Dasgupta A. Structure and function of a small RNA that selectively inhibits internal ribosome entry site-mediated translation. *Nucleic Acids Res* 1999; **27**: 563-572

Edited by Gupta KM and Wang XL

• *H pylori* •

# Low eradication rate of *Helicobacter pylori* with triple 7-14 days and quadruple therapy in Turkey

Yuksel Gumurdulu, Ender Serin, Birol Özer, Fazilet Kayaselcuk, Kursat Ozsahin, Arif Mansur Cosar, Murat Gursay, Gurden Gur, Ugur Yilmaz, Sedat Boyacioglu

**Yuksel Gumurdulu, Ender Serin, Birol Özer, Arif Mansur Cosar**, Faculty of Medicine, Baskent University, Department of Gastroenterology, Adana Teaching and Medical Research Center, Adana, Turkey

**Fazilet Kayaselcuk**, Department of Pathology, Adana Teaching and Medical Research Center, Adana, Turkey

**Kursat Ozsahin**, Department of Family Physician, Adana Teaching and Medical Research Center, Adana, Turkey

**Murat Gursay, Gurden Gur, Ugur Yilmaz, Sedat Boyacioglu**, Faculty of Medicine, Baskent University, Department of Gastroenterology, Ankara Hospital, Ankara, Turkey

**Correspondence to:** Yuksel Gumurdulu, MD, Ba<sup>o</sup> kent Üniversitesi Tıp Fakültesi, Adana Uygulama ve Ara<sup>o</sup> tırma Merkezi, Dadalođlu Mahallesi, 39 Sokak, No: 6, 01250 Adana, Turkey. yukselgumurdulu@hotmail.com

**Telephone:** +90-322-3272727 **Fax:** +90-322-3271273

**Received:** 2003-10-08 **Accepted:** 2003-11-12

## Abstract

**AIM:** The eradication rate of *Helicobacter pylori* (*H pylori*) shows variation among countries and regimens of treatment. We aimed to study the eradication rates of different regimens in our region and some factors affecting the rate of eradication.

**METHODS:** One hundred and sixty- four *H pylori* positive patients (68 males, 96 females; mean age: 48±12 years) with duodenal or gastric ulcer without a smoking history were included in the study. The patients were divided into three groups according to the treatment regimens. Omeprazole 20 mg, clarithromycin 500 mg, amoxicillin 1 g were given twice daily for 1 week (Group I) and 2 weeks (Group II). Patients in Group III received bismuth subsitrate 300 mg, tetracycline 500 mg and metronidazole 500 mg four times daily in addition to Omeprazole 20 mg twice daily. Two biopsies each before and after treatment were obtained from antrum and corpus, and histopathologically evaluated. Eradication was assumed to be successful if no *H pylorus* was detected from four biopsy specimens taken after treatment. The effects of factors like age, sex, *H pylori* density on antrum and corpus before treatment, the total *H pylori* density, and the inflammation scores on the rate of *H pylori* eradication were evaluated.

**RESULTS:** The overall eradication rate was 42%. The rates in groups II and III were statistically higher than that in group I ( $P<0.05$ ). The rates of eradication were 24.5%, 40.7% and 61.5% in groups I, II and III, respectively. The eradication rate was negatively related to either corpus *H pylori* density or total *H pylori* density ( $P<0.05$ ). The median age was older in the group in which the eradication failed in comparison to that with successful eradication (55 yr vs 39 yr,  $P<0.001$ ). No correlation between sex and *H pylori* eradication was found.

**CONCLUSION:** Our rates of eradication were significantly lower when compared to those reported in literature. We

believe that advanced age and high *H pylori* density are negative predictive factors for the rate of *H pylori* eradication.

Gumurdulu Y, Serin E, Özer B, Kayaselcuk F, Ozsahin K, Cosar AM, Gursay M, Gur G, Yilmaz U, Boyacioglu S. Low eradication rate of *Helicobacter pylori* with triple 7-14 days and quadruple therapy in Turkey. *World J Gastroenterol* 2004; 10(5): 668-671  
<http://www.wjgnet.com/1007-9327/10/668.asp>

## INTRODUCTION

Eradication treatment for *H pylori* infection has been generally accepted since the relation between *H pylori* and peptic ulcer disease was established<sup>[1,2]</sup>. Furthermore, this treatment approach has gained importance since the eradication of *H pylori* was found to reduce the recurrence of duodenal and gastric ulcer<sup>[3,4]</sup>. The eradication rate of *H pylori* has reached high levels with combined use of antibiotics and proton pump inhibitors (PPIs), being 60-90% in Turkey<sup>[5]</sup>. Different treatment protocols have been used for *H pylori* eradication. The rate of *H pylori* eradication was higher than 85% with the combination of drugs consisting of two antibiotics and a PPI<sup>[6]</sup>. We aimed to study the rates of *H pylori* eradication in Cukurova region with regard to different treatment regimens and the effects of patient age, sex, and *H pylori* density on *H pylori* eradication rates.

## MATERIALS AND METHODS

One hundred and 64 patients (68 males, 96 females; mean age 48±12 yr, range 17-78 yr) with gastric or duodenal ulcer and *H pylori* detected at endoscopy were included in the study. Two biopsies from antrum and two from corpus were taken. *H pylori* density and gastric inflammation in both antrum and corpus were assessed based on Sydney classification (normal=0, mild=1, moderate=2, marked=3)<sup>[7]</sup>. The patients were randomly divided into three groups and each group was treated with one of the protocols as follows. Group I received Omeprazole (O) 20 mg, Clarithromycin (C) 500 mg, amoxicillin (A) 1 000 mg, twice daily for 7 d. Group II received the same drugs as in group I for 14 d. Group III received Omeprazole 20 mg twice daily, Bismute subsitrate (BS) 300 mg 4 times daily, tetracycline (T) 500 mg 4 times daily, metronidazole (M) 500 mg 4 times daily for 10 d. There were 53, 59 and 52 patients in the 3 groups, respectively. Antibiotics, PPIs, and H<sub>2</sub> receptor blockers were used for at least one month. Smoking, pregnancy and lactation, past history of gastric surgery, renal or liver failure, diabetes mellitus and irregular use of drugs in the eradication regimens were accepted as exclusion criteria. Endoscopy was repeated and two biopsies each from corpus and antrum were obtained after 45-60 d of treatment. Eradication was accepted to be successful if *H pylori* were not found in any of the 4 samples. Total bacterial density was calculated semi quantitatively by addition of antrum and corpus *H pylori* density<sup>[8]</sup>. None of the patients had atrophy in histologic evaluation. The relations of *H pylori* densities in

different locations before treatment, age, and sex with the rate of *H pylori* eradication were analyzed.

### Statistical analysis

Data were expressed as medians with interquartiles. Mann-Whitney-*U* test or  $\chi^2$  test was used to assess significant differences between values in various groups of patients.  $P < 0.05$  was considered statistically significant. Data were analyzed using the SPSS for Windows (version 9.05; SPSS, Inc., Chicago, Illinois, USA).

## RESULTS

Out of 164 patients who completed the study, 69 (42%) had eradication of *H pylori* infection, and 151 (91.4%) showed ulcer healing. Eradication could not be achieved in 10 of 13 patients without ulcer healing. The eradication rate in group I, II, and III was 24.5% (13/53), 40.6% (24/59), and 61.5% (32/52), respectively. The rate of eradication in group III was higher than group I and II ( $P < 0.03$ ). There was a difference between groups I and II, which did not reach statistical significance ( $P = 0.07$ ). The ulcer cure was 45/53, 55/59, and 51/52, respectively in the 3 groups. *H pylori* density in antrum before treatment or severity of inflammation was not statistically related to its eradication. Corpus and total *H pylori* densities were higher in patients who failed the eradication treatment when compared to those who showed successful eradication ( $P < 0.01$  and  $P < 0.05$ , respectively for corpus and total densities) (Table 1). The median patient age was higher in the group in which eradication failed. (55 vs 39 yrs,  $P < 0.001$ ). The distribution of age, sex and *H pylori* density of corpus in the patient groups were summarized in Table 2.

**Table 1** Rates of *H pylori* eradication and ulcer healing in various treatment regimens

Group	n	Age	F/M	Eradication (%)	Ülcer healing <sup>d</sup> (%)
I	53	49±12	30/23	13 (24.5) <sup>a,b</sup>	45 (84.9)
II	59	48±11	35/24	24 (40.7) <sup>c</sup>	55 (93.2)
III	52	45±12	31/21	32 (61.5)	51 (98)
Total	164	48±12	96/68	69 (42)	151 (92.1)

<sup>a</sup> $P = 0.07$  vs Group II; <sup>b</sup> $P < 0.01$  vs Group III; <sup>c</sup> $P < 0.03$  vs Group III;

<sup>d</sup> $P = \text{N.S.}$  among the three groups.

## DISCUSSION

The discovery of *H pylori* by Marshall and Warren has been considered as a revolution<sup>[9]</sup>. As the role of this microorganism in gastric pathologies is fully understood, the treatment principles of some of the gastro-duodenal lesions have been changed<sup>[10]</sup>.

Nowadays, *H pylori*-positive peptic ulcer disease is accepted as an infectious process, and combination of drugs,

including the same antibiotics were used in all of the treatment protocols<sup>[11-13]</sup>. No standard therapy for *H pylori* eradication has been underlined. An ideal *H pylori* treatment must be safe, cheap, easy and tolerable with more than an 80% eradication rate and must have a low rate of antibiotic resistance<sup>[14,15]</sup>.

PPIs are commonly used in the combined regimens as a result of significant *in vivo* and *in vitro* effects on *H pylori*. Many treatment regimens including PPIs were still hard to eradicate *H pylori*<sup>[16-19]</sup>.

In Europe and in the USA despite resistance to metronidazole, clarithromycin, and tetracycline was 30-40%, 2-10% and <1%, respectively, the eradication rate of the combined treatment with clarithromycin, PPI and amoxicillin or metronidazole for 7-14 d was 87-100% in metronidazole sensitive group and 57-88% in metronidazole resistant group. In 10-12 d of PPI-A-C regimens, 88-96% eradication rate was maintained in clarithromycin sensitive group and 50% in the resistant group. These findings showed the importance of antibiotic resistance in unsuccessful eradication trials<sup>[20]</sup>. Several studies, in which the antibiotic resistance was analyzed as one of the independent parameters that can be predictors of eradication failure, supported the same suggestion<sup>[21-23]</sup>. In Turkey, Boyanova *et al* found the resistance to M 37.9%, to C 9.5%, and to A 0.9%, respectively. The double drug resistance (M+C) was 6.1%<sup>[24]</sup>. In a former study we found C and M resistance rates were 5.2% (1/19) and 36.8% (7/19) in a group of 19 patients (data unpublished). These observations suggested that antibiotic resistance could not explain completely the low eradication rate as we observed in the present study. The resistance in our patients was similar to that detected in the Western world whereas the rate of eradication was markedly lower when compared to Western population.

The effect of combination therapies on *H pylori* eradication rates varied according to the differences in treatment duration. Some studies which showed that 14 d trials were more successful than 7 d trials, whereas other studies showed no difference. In our study the rate of *H pylori* eradication was higher in prolonged treatment but did not reach any statistical significance<sup>[25]</sup>.

The reported higher rates of *H pylori* eradication with either OCA or OCM combination in early studies in our country as compared to recent ones suggested the development of resistance was due to irregular and unsubscribed use of antibiotics in our population<sup>[17]</sup>. The possible differences in the production process of drugs by various manufacturers may play a role in the bioavailability of active compounds. We could not find any study regarding to this issue. However, Kim *et al.* from South Korea have found similar benefits in *H pylori* eradication rate of three drug combinations with two different Omeprazole preparations<sup>[26]</sup>. If similar studies can be made for antibiotics, questions on this matter can be answered.

Patient incompliance to treatment is another factor contributing to eradication failure. Graham *et al* have found that the eradication rate was about 96% in patients who used

**Table 2** The distribution of age, sex and *H pylori* density of corpus in the patient groups

Group	Eradicated				Non-eradicated			
	n	Age <sup>a</sup>	F/M	Density	n	Age <sup>a</sup>	F/M	Density
I	13	35.3 ± 11.7 <sup>a</sup>	9/4	1.31 ± 0.95 <sup>b</sup>	40	54.0 ± 9.1 <sup>a</sup>	21/19	2.17 ± 0.87 <sup>b</sup>
II	24	40.04 ± 9.3 <sup>a</sup>	13/11	1.92 ± 0.8 <sup>b</sup>	35	54.94 ± 8.8 <sup>a</sup>	21/14	2.37 ± 0.77 <sup>b</sup>
III	32	40.03 ± 11.6 <sup>a</sup>	20/12	1.91 ± 0.69 <sup>b</sup>	20	55.15 ± 9.0 <sup>a</sup>	12/8	2.35 ± 0.75 <sup>b</sup>
Total	69	39.14 ± 10.9 <sup>a</sup>	42/27	1.8 ± 0.83 <sup>b</sup>	95	54.61 ± 8.9 <sup>a</sup>	54/41	2.28 ± 0.79 <sup>b</sup>

<sup>a</sup> $P < 0.001$ , vs noneradicated in each group for age, <sup>b</sup> $P < 0.05$ , vs noneradicated in each group for *H pylori* corpus density.

60% or more of their drugs and 69% in those who used less than 60% of drugs<sup>[27]</sup>. We tried to eliminate this factor via good questioning and follow-up throughout the study. Patient incompliance did not seem to be a determinant factor for *H pylori* eradication failure in our study.

The response to combination of four drugs was around 87-100% in several studies. Nelson *et al.* found that the ulcer cure and eradication rate of *H pylori* with BSOMT in a 2 d and 7 d regimen were 95.7% and 76.1%, and 98% and 100%, respectively<sup>[11]</sup>. In studies using BSTMO combination, the eradication rate of *H pylori* was 96% in the metronidazole sensitive group and 82% in the metronidazole resistant group<sup>[28]</sup>. The rate of *H pylori* eradication with the same protocol in our study was much lower compared to previous trials, but significantly higher than two other protocols we used. This shows the necessity for studying some geographic factors.

Smoking; one of the parameters of antibiotic resistance in literature, was restrained during the inclusion phase of this study. We studied two other factors associated with *H pylori* eradication failure, namely the grade of *H pylori* density and gastritis. Our findings suggested that the eradication was more difficult in patients who had high total or corpus *H pylori* density. Georgopoulos *et al* found no relation between eradication and *H pylori* density or the severity of gastritis<sup>[21]</sup>. Yang *et al* reported a lower total bacterial density in the eradicated group<sup>[8]</sup>. In our study corpus and total (sum of *H pylori* density in antrum and corpus) *H pylori* densities were higher in patients with eradication failure compared to the successfully eradicated group. Consequently, the factors affecting *H pylori* density can be expected to affect the eradication rate. Some studies on this issue showed that some factors related with host or bacteria could affect *H pylori* density in gastric mucosa<sup>[21,29-31]</sup>.

The patients' lifetime exposure to several kinds of antibiotics could cause resistance and this might partly explain the difference noted in the age groups with respect to *H pylori* eradication rate<sup>[32]</sup>.

Another factor that can contribute to the resistance to antibiotic treatment is intracellular settling of bacteria. The examples of this fact were some chronic infections such as tuberculosis and brucellosis for which a long term use of antibiotics was required<sup>[33,34]</sup>. The difficulty in eradication treatment of *H pylori* may in part be due to intracellular location of these bacteria since *H pylori* has been shown to penetrate into cells in cell cultures<sup>[35]</sup>. This suggestion was firmly supported when 14 d regimens were shown to be more effective than 7 d regimens as reported in this and previous studies.

The rate of eradication we encountered was significantly lower than those in literature, which suggests further studies concerning the mechanisms underlying the eradication failure in our community should be designed.

## REFERENCES

- Ikeda S, Tamamuro T, Hamashima C, Asaka M. Evaluation of the cost-effectiveness of *Helicobacter pylori* eradication triple therapy vs conventional therapy for ulcers in Japan. *Aliment Pharmacol Ther* 2001; **15**: 1777-1785
- Malfertheiner P, Megraud F, O' Morain C, Hungin AP, Jones R, Axon A, Graham DY, Tytgat G. Current concepts in the management of *Helicobacter pylori* infection—the Maastricht 2-2000 Consensus Report. *Aliment Pharmacol Ther* 2002; **16**: 167-180
- Marzio L, Cellini L, Angelucci D. Triple therapy for 7 days vs. triple therapy for 7 days plus omeprazole for 21 days in treatment of active duodenal ulcer with *Helicobacter pylori* infection. A double blind placebo controlled trial. *Dig Liver Dis* 2003; **35**: 20-23
- Leodolter A, Kulig M, Brasch H, Meyer-Sabellek W, Willich SN, Malfertheiner P. A meta-analysis comparing eradication, healing and relapse rates in patients with *Helicobacter pylori*-associated gastric or duodenal ulcer. *Aliment Pharmacol Ther* 2001; **15**: 1949-1958
- Tytgat GN. Review article: treatments that impact favourably upon the eradication of *Helicobacter pylori* and ulcer recurrence. *Aliment Pharmacol Ther* 1994; **8**: 359-368
- Bhasin DK, Sharma BC, Ray P, Pathak CM, Singh K. Comparison of Seven and Fourteen Days of Lansaprazole, Claritromycin and amoxicillin Therapy for Eradication of *Helicobacter pylori*: A Report from India. *Helicobacter* 2000; **5**: 84-87
- Dixon MF, Genta RM, Yardley JH, Correa P. Classification and Grading of Gastritis. The Updated Sydney system. *Am J Surg Pathol* 1996; **20**: 1161-1181
- Yang HB, Sheu BS, Su IJ, Chien CH, Lin XZ. Clinical Application of Gastric Histology to Monitor Treatment of Dual Therapy in *H pylori* Eradication. *Dig Dis Sci* 1997; **42**: 1835-1840
- Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1983; **1**: 1273-1275
- World Health Organization. International agency for research on cancer. Infection with *Helicobacter pylori*. Schistosomes, Liver flukes and *Helicobacter pylori*. Lyon: IARC 1994: 172-202
- Hopkins RJ, Girardi LS, Turney EA. Relationship between *Helicobacter pylori* eradication and reduced duodenal and gastric ulcer recurrence: a review. *Gastroenterology* 1996; **110**: 1244-1252
- Peitz U, Hackelsberger A, Malfertheiner P. A practical approach to patients with refractory *Helicobacter pylori* infection, or who are re-infected after standard therapy. *Drugs* 1999; **57**: 905-920
- Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **16**: 1311-1315
- Working Party of the european *Helicobacter pylori* study group. Guidelines for clinical trials in *Helicobacter pylori* infection. *Gut* 1997; **41**(Suppl 2): S1-9
- Soll AH. Consensus conference. Medical treatment of peptic ulcer disease. Practice guidelines. Practice Parameters Committee of the American College of Gastroenterology. *JAMA* 1996; **275**: 622-628
- Taskin V, Özyılkan E, Aydın A, Çetin F, Eskioğlu E, Köseoğlu T. Can resistant *Helicobacter pylori* infection be estimated histologically: Effects of gastric histology on eradication rates of *Helicobacter pylori* infection. *The Turk J Gastroenterol* 2001; **12**: 126-129
- Aydın A, Ersöz G, Tunçyürek M, Cavusoglu H. One-week triple therapies for *Helicobacter pylori* eradication. *The Turk J Gastroenterol* 1998; **9**: 40-45
- Iwahi T, Satoh H, Nakao M, Iwasaki T, Yamazaki T, Kubo K, Tamura T, Imada A. Lansaprazole, a novel benzimidazole proton pump inhibitor, and its related compounds have selective activity against *Helicobacter pylori*. *Antim Agents Chem* 1991; **35**: 490-496
- Hunt RH. pH and *H pylori*-gastric acid secretion and *Helicobacter pylori*. Implications for ulcer healing and eradication the organism. *Am J Gastroenterol* 1993; **88**: 481-483
- Glupczynski Y. Antimicrobial resistance in *Helicobacter pylori*: a global overview. *Acta Gastroenterol Belg* 1998; **61**: 357-366
- Georgopoulos SD, Ladas SD, Karatapanis S, Mentis A, Spiliadi C, Artikis V, Raptis SA. Factors that may Affect Treatment Outcome of Triple *Helicobacter pylori* Eradication Therapy with Omeprazole, Amoxicillin, and Clarithromycin. *Dig Dis Sci* 2000; **45**: 63-67
- Huang JQ, Hunt RH. Treatment after failure: the problem of 'non-responders'. *Gut* 1999; **45**(Suppl1): 140-144
- Kim JJ, Reddy R, Lee M, Kim JG, El-Zaatari FA, Osato MS, Graham DY, Kwon DH. Analysis of metronidazole, claritromycin and tetracycline resistance of *Helicobacter pylori* isolated from Korea. *J Antim Chem* 2001; **47**: 459-461
- Boyanova L, Mentis A, Gubina M, Rozynek E, Gosciniak G, Kalenic S, Goral V, Kupcinskas L, Kantarceken B, Aydın A, Archimandritis A, Dzierzanowska D, Vcev A, Ivanova K, Marina M, Mitov I, Petrov P, Ozden A, Popova M. The status of antimicrobial resistance of *Helicobacter pylori* in eastern Europe. *Clin Microbiol Infect* 2002; **8**: 388-396
- Forne M, Viver JM, Esteve M, Fernandez-Banares F, Lite J, Espinos JC, Quintana S, Salas A, Garau J. Randomize clinical trial comparing two one week triple therapy regimens for the eradication of *Helicobacter pylori* infection and duodenal ulcer healing. *Am J Gastroenterol* 1998; **93**: 35-38



- 26 **Kim HS**, Lee DK, Kim KH, Jeong YS, Kim JW, Seo JI, Baik SK, Kwon SO, Cho MY. Comparison of the efficacy and safety of different formulations of omeprazole-based triple therapies in the treatment of *Helicobacter pylori*-positive peptic ulcer. *J Gastroenterol* 2001; **36**: 96-102
- 27 **Graham DY**, Lew GM, Malaty HM, Evans DG, Evans DJ Jr, Klein PD, Alpert LC, Genta RM. Factors influencing eradication of *Helicobacter pylori* with triple therapy. *Gastroenterology* 1992; **102**: 493-496
- 28 **Kung NN**, Sung JJ, Yuen NW, Ng PW, Wong KC, Chung EC, Lim BH, Choi CH, Li TH, Ma HC, Kwok SP. Anti-*Helicobacter pylori* Treatment in Bleeding Ulcers: Randomized Controlled Trial Comparing 2-day versus 7-Day bismuth Quadruple Therapy. *Am J Gastroenterol* 1997; **92**: 438-441
- 29 **Megraud F**. Resistance of *Helicobacter pylori* to antibiotics: the main limitation of current proton-pump inhibitor triple therapy. *Eur J Gastroenterol Hepatol* 1999; **11**: (Suppl 2): S35-37
- 30 **Kamada T**, Haruma K, Komoto K, Mihara M, Chen X, Yoshihara M, Sumii K, Kajiyama G, Tahara K, Kawamura YM. Effect of smoking and histological gastritis severity on the rate of *H pylori* eradication with omeprazole, amoxicillin, and clarithromycin. *Helicobacter* 1999; **4**: 204-210
- 31 **Russo A**, Maconi G, Spinelli P, Felice GD, Eboli M, Andreola S, Ravagnani F, Settesoldi D, Ferrari D, Lombardo C, Bertario L. Effect of lifestyle, smoking, and diet on development of intestinal metaplasia in *H pylori*-positive subject. *Am J Gastroenterol* 2001; **96**: 1402-1408
- 32 **Huang JQ**, Hunt RH. Review: eradication of *Helicobacter pylori*. Problems and recommendations. *J Gastroenterol Hepatol* 1997; **12**: 590-598
- 33 **Raviglione MC**, O'Brien RJ. Tuberculosis. in Fauci A.S.ed. *Harrison's principles of internal medicine*. **14<sup>th</sup>** ed. New York: McGraw-Hill Co 1998: 1004-1014
- 34 **Madkour M**. Brucellosis in Fauci A.S.ed. *Harrison's principles of internal medicine*. **14<sup>th</sup>** ed. New York: McGraw-Hill co 1998: 1004-1014
- 35 **Bjorkholm B**, Zhukhovitsky V, Lofman C, Hulten K, Enroth H, Block M, Rigo R, Falk P, Engstrand L. *Helicobacter pylori* entry into human gastric epithelial cells: A potential determinant of virulence, persistence, and treatment failures. *Helicobacter* 2000; **5**: 148-154

Edited by Wang XL Proofread by Zhu LH

• *H pylori* •

# Relationship of gastric *Helicobacter pylori* infection to Barrett's esophagus and gastro-esophageal reflux disease in Chinese

Jun Zhang, Xiao-Li Chen, Kang-Min Wang, Xiao-Dan Guo, Ai-Li Zuo, Jun Gong

**Jun Zhang, Jun Gong, Ai-Li Zuo, Xiao-Dan Guo**, Department of Gastroenterology, Second Hospital, Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China

**Xiao-Li Chen, Kang-Min Wang**, Department of Pathology, Second Hospital of Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China

**Correspondence to:** Dr. Jun Zhang, Department of Gastroenterology, Second Hospital, Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China. jun3z@163.com

**Telephone:** +86-29-7678009

**Received:** 2003-09-06 **Accepted:** 2003-10-27

## Abstract

**AIM:** To evaluate the relationship of *Helicobacter pylori* infection to reflux esophagitis (RE), Barrett's esophagus (BE) and gastric intestinal metaplasia (IM).

**METHODS:** RE, BE and gastric IM were determined by upper endoscopy. Patients were divided into 2 groups; those with squamocolumnar junction (SCJ) beyond gastroesophageal junction (GEJ)  $\geq 3$  cm (group A), and those with SCJ beyond GEJ  $< 3$  cm (group B). Biopsy specimens were obtained endoscopically from just below the SCJ, gastric antrum along the greater and lesser curvature. Pathological changes and *H pylori* infection were determined by HE staining, Alcian blue staining and Giemsa staining.

**RESULTS:** The prevalence of *H pylori* infection was 46.93%. There was no difference in the prevalence between males and females. The prevalence of *H pylori* infection decreased stepwise significantly from RE grade I to III. There was no difference in the prevalence between the two groups, and between long-segment and short-segment BE. In distal stomach, prevalence of *H pylori* infection was significantly higher in patients with IM than those without IM.

**CONCLUSION:** There is a protective role of *H pylori* infection to GERD. There may be no relationship between *H pylori* infection of stomach and BE. *H pylori* infection is associated with the development of IM in the distal stomach.

Zhang J, Chen XL, Wang KM, Guo XD, Zuo AL, Gong J. Relationship of gastric *Helicobacter pylori* infection to Barrett's esophagus and gastro-esophageal reflux disease in Chinese. *World J Gastroenterol* 2004; 10(5): 672-675

<http://www.wjgnet.com/1007-9327/10/672.asp>

## INTRODUCTION

The incidence of adenocarcinoma in the esophagus and gastroesophageal junction (GEJ) is increasing, whereas the incidence of distal gastric cancer is falling for the two decades in North America, Europe, Japan and China. In China, the incidence of adenocarcinoma at the GEJ is increasing even more significantly<sup>[1-3]</sup>.

The adenocarcinomas at the esophagus and GEJ differ

from those in the stomach<sup>[4]</sup>. They share epidemiological characteristics with each other, and often originate from segments of Barrett's esophagus (BE). It has therefore been proposed that both of them can be called "esophagocardia adenocarcinoma"<sup>[5]</sup>. BE is a well-defined premalignant condition for esophageal adenocarcinoma and most of adenocarcinomas at GEJ<sup>[6,7]</sup>. Neoplastic progression of BE has been shown to involve multiple steps with intestinal metaplasia and dysplasia serving as histopathologic markers<sup>[5]</sup>. It is considered that the absence of specialized intestinal metaplasia in many patients with adenocarcinoma at the GEJ may be due to the complete replacement of the metaplastic epithelium by the tumor, and in these tumors, IM usually is confined to ultrashort segments that may easily be overgrown by the tumor<sup>[8]</sup>.

Gastro-esophageal reflux disease (GERD) can give rise to BE, and reflux symptoms are important indicators that a patient is at risk of having Barrett's metaplasia. Recently, interest has focused on the relationship between *H pylori* infection and GERD as well as BE, but controversial findings have been obtained. Several retrospective studies have examined the association of Barrett's adenocarcinoma with gastric *H pylori* infection, yielding discordant results. It has been known that the prevalence of *H pylori* infection in China is high. It is necessary to clarify whether *H pylori* infection is the causally associated with BE or whether it has a protective effect on BE. In addition, further investigations are also required to clarify the relationship between *H pylori* infection and GERD. Therefore, the aim of this prospective study was to evaluate the relationship between gastric *H pylori* infection and reflux esophagitis (RE), BE, and gastric IM in China.

## MATERIALS AND METHODS

### Patients

Consecutive patients undergoing esophagogastroendoscopy at Second Hospital, Xi'an Jiaotong University, Xi'an, China from August 1, 2000 to the end of August 1, 2001 were included in the study. Exclusion criteria included previous gastric or esophagus resection, contraindication to performing biopsies, prior history of *H pylori* eradication therapy, and/or use of bismuth-containing compounds or antibiotics within the previous 4 weeks. The study was approved by the Ethics Committee of the hospital, and informed consents were obtained from all patients before entry.

### Endoscopy and biopsy

Endoscopy was performed in a standardized manner by experienced endoscopists. The appearance of the squamocolumnar junction (SCJ) was carefully studied in the prograde view after insufflation of air and after retroversion in the stomach. According to the length from the GEJ to SCJ, patients were divided into two groups; those with velvety red gastric-like mucosa lining the distal esophagus for 3 cm or over (group A) and those with velvety red gastric-like mucosa lining the distal esophagus for less than 3 cm (group B). Endoscopic esophagitis was graded as I, mucosal erythema; II, non-circumferential mucosal breaks or erosions; III,

circumferential erosion or ulcer.

Four-quadrant biopsies were taken from the area immediately distal to the SCJ. Additional targeted biopsies were also taken from erosions, nodules or ulcers. For assessment of *H pylori* status biopsies were taken from antral greater curvature (two), and lesser curvature (two).

Biopsy specimens were fixed in 40 g/L buffered formaldehyde, embedded in paraffin, serially sectioned, and then stained with hematoxylin and eosin. BE was defined as the presence of distended, barrel-shaped goblet cells, indicative of intestinal metaplasia<sup>[6,31,32]</sup>, which was further confirmed by staining with Alcian blue pH 2.5.

In addition, BE was divided into long-segment Barrett's esophagus (LSBE, the segments of IM more than or equal to 3 cm in length) and short-segment Barrett's esophagus (SSBE, the segment of IM less than 3 cm in length).

The presence of gastric *H pylori* infection was defined when one or more of Giemsa-stained gastric biopsy specimens demonstrated typical *H pylori*-like organisms.

The gastric IM was defined by the presence of barrel-shaped goblet cells.

### Statistical analysis

Statistical analyses were performed using the  $\chi^2$  test.

## RESULTS

Altogether, 391 patients were recruited. Of these patients, 253 had esophageal disorders; 103 with RE (39 grade I, 35 grade II and 29 grade III), 120 with BE (26 LSBE and 94 SSBE), 12 with dysplasia (seven low-grade and five high-grade), 17 with adenocarcinoma at the GEJ and one with adenocarcinoma at lower esophagus (Table 1). Males were more likely to have esophageal disorders than females. The average age ranged from 52.41 to 62.64 years old, with and increased progressively from RE→BE→LGD→HGD→adenocarcinoma.

**Table 1** Clinic features of study population

	No.	Mean age (yr)	Male: Female
No. Of patients	391	52.41	211:180
RE I	39	52.12	26:13
II	35	53.67	26:9
III	29	55.56	24:5
BE SSBE	94	54.71	62:32
LSBE	26	58.66	20:6
Low-grade dysplasia	7	59.57	5:2
High-grade dysplasia	5	62.00	3:2
Adenocarcinoma at GEJ	17	62.64	14:3

Status of *H pylori* infection was available for 375 patients. The prevalence of *H pylori* infection was 46.93% (176/375). There was no significant difference in the prevalence between males and females (males, 48.71% and females, 45.00%) ( $P>0.05$ ) (Table 2). The prevalence of *H pylori* infection in Group A was 41.84% (41/98), which was slightly lower than that 48.73% (135/277) in group B ( $P>0.05$ ). The prevalence of *H pylori* infection decreased stepwise significantly from RE Grade I (51.72%), grade II (28.57%) to grade III (20.68%) ( $P<0.05$ ) (Table 2).

The prevalence of IM in group A (LSBE) (26.53%) was slightly lower than that in group B (SSBE) (33.94%) ( $P>0.05$ ). In groups A and B the prevalence of *H pylori* infection (46.15% and 51.06%, respectively) in patients with IM was slightly higher than that (40.27% and 47.54%, respectively) in those without IM (both  $P>0.05$ ) (Table 3). The prevalence of *H pylori* infection of LSBE (46.15%) is slightly lower than that of SSBE

(51.06%) ( $P>0.05$ ). However, in the distal stomach, the prevalence of *H pylori* infection in patients with IM (56.29%) was significantly higher than that in those without IM (37.89%) ( $P<0.05$ ) (Table 3).

**Table 2** Comparisons of *H pylori* status among patients with sex, length of SCJ, grade of RE

	No.	Hp <sup>+</sup> (%)	Hp (%)	P
No. Of patients	375	176 (46.93)	199 (53.06)	
Male: Female	195:180	95: 81	100:99	0.534
Group A	98	41 (41.84)	57 (58.16)	0.239
Group B	277	135 (48.73)	142 (51.26)	
RE I	29	15 (51.72)	14 (48.28)	0.032
II	35	10 (28.57)	25 (41.43)	
III	29	6 (20.68)	23 (79.31)	

**Table 3** Comparisons of *H pylori* status between patients with IM in LSBE, SSBE and distal stomach

IM	Group A		Group B		Distal stomach	
	+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
HP+	12 (46.15)	29 (40.27)	48 (51.06)	87 (47.54)	76 (56.29)	97 (37.89)
HP-	14 (53.84)	43 (59.72)	46 (48.93)	96 (52.45)	59 (43.70)	159 (62.11)
Total	26	72	94	183	135	256

Comparison of *H pylori* status between LSBE (IM+ in group A) and SSBE (IM+ in group B),  $P=0.658$ , ( $\chi^2=0.196$ ); Comparison of *H pylori* status between IM "+" and IM "-" in group A,  $P=0.603$ , ( $\chi^2=0.271$ ); Comparison of *H pylori* status between IM "+" and IM "-" in group B,  $P=0.579$ , ( $\chi^2=0.308$ ); Comparison of *H pylori* status between IM "+" and IM "-" in distal stomach,  $P=0.000$ , ( $\chi^2=12.14$ ).

## DISCUSSION

Since the isolation of *H pylori* from gastric mucosa by Warren and Marshall in 1983, there has been renewed interest in a possible bacterial cause of upper gastrointestinal diseases<sup>[9]</sup>. *H pylori* is now widely accepted as a major cause of antral gastritis and peptic ulcer disease<sup>[10,11]</sup>. Epidemiological evidence for an association with gastric carcinoma has also been reported<sup>[12,13]</sup>. In addition, gastric mucosa-associated lymphoid tissue (MALT) lymphoma has been linked to *H pylori* infection<sup>[14,15]</sup>. The relationship of *H pylori* to GERD and BE is less clear. Some groups have reported a lower prevalence of *H pylori* infection in individuals with GERD, and have postulated that infection may reduce the risk of reflux esophagitis<sup>[16-18]</sup>. However, other epidemiological studies have found little or no association between *H pylori* infection and GERD<sup>[19-21]</sup>. With regard to Barrett's esophagus the majority of studies have found no association with *H pylori* infection<sup>[22,23]</sup>. However, there is also evidence to the contrary<sup>[18]</sup>. Our prospective study tried to evaluate the relation between gastric *H pylori* infection and RE, BE, and gastric IM in Chinese.

*H pylori* infection can be diagnosed by a variety of noninvasive or invasive tests. Histological examination with special staining of gastric biopsy specimens is accepted as the gold standard for *H pylori* diagnosis<sup>[24,25]</sup>. Gastric antrum is the most common place for *H pylori* colonization<sup>[26,27]</sup>. Modified Giemsa staining, which has been shown to have a high specificity and sensitivity<sup>[25,28]</sup>, was used to detect *H pylori* status in this study.

In early studies, BE was defined as the presence of specialized IM in a columnar-lined mucosa encompassing more than 3 cm proximal to GEJ or a LSBE<sup>[29]</sup>. Any columnar-lined mucosa less than 3 cm above the GEJ was thought to be a

normal variant. However studies over the past years have indicated that there is a spectrum of involvement that includes the distal 3 cm of esophagus or a SSBE<sup>[30,31]</sup>. What is important is the presence of IM relating to adenocarcinoma. It has been shown that a patient with LSBE has a higher risk to develop dysplasia or cancer<sup>[32]</sup>. It also has been shown that the development of LSBE is more closely related to gastroesophageal reflux<sup>[33]</sup>. Therefore, in our study, patients were divided into two groups; those with a velvety red gastric-like mucosa lining the distal esophagus for 3 cm or over and those with a velvety red gastric-like mucosa lining the distal esophagus for less than 3 cm. Because esophageal hiatal hernia is often complicated by GERD and BE, many researchers think that to define an esophageal hiatal hernia, the presence of a velvety red gastric-like mucosa lining the distal esophagus for 2 cm should be considered as normal. In our study, therefore, we referred to this definition; the length from the GEJ to SCJ for esophageal hiatal hernia reduced 2 cm<sup>[34,35]</sup>.

Altogether, 391 patients were evaluated over the course of the study (Table 1). The presence of IM was confirmed in 26 cases of the 98 patients suspected of having LSBE, and in 94 cases of the 277 patients suspected of having SSBE. Seven BE cases with dysplasia, 103 RE and 17 adenocarcinoma at GEJ were diagnosed. The average age of patients increased gradually with the sequence of RE, BE, LGD, HGD and adenocarcinoma at GEJ from 52.41 to 62.64 years old. Males were more likely to have the diseases. Cameron *et al* reported that the age after 40 had a high incidence of BE, and development of adenocarcinoma from BE required about 20 years<sup>[36]</sup>.

Of the entire study population, 375 subjects received histological examination for *H pylori* infection, with a prevalence of *H pylori* infection of 46.93%. There was no significant difference between males and females. These results are similar to the report by Hui *et al* about *H pylori* infection in the same area of China<sup>[37]</sup>.

The first change in BE, is the replacement of the normal stratified epithelium with metaplastic columnar epithelium in the distal esophagus, making the SCJ rising upward above the GEJ. It is accepted that BE is an acquired condition, and is related to gastro-esophageal reflux. An excellent overview and hypothesis detailing the role of *H pylori* infection in the pathogenesis of duodenal ulcer, gastric cancer, and GERD has been given by Graham and Yamaoka<sup>[38,39]</sup>. *H pylori* infection has been shown to decrease acid secretion in patients with body-predominant *H pylori* colonization<sup>[40]</sup>. With less acid production, the offensive potency of the refluxate may be reduced. An additional mechanism could be neutralization of acid by ammonia produced by *H pylori*, with subsequent reduction in intragastric acid load and in the reflux of acid into the esophagus, as proposed by Bercik *et al*<sup>[41]</sup>. Ammonia may also promote protective adaptation of the esophageal mucosa<sup>[42]</sup>. So, if *H pylori* infection can decrease the offensive potency of the refluxate, we could infer indirectly whether *H pylori* infection has the protective effect in GERD and BE by comparing the RE grade and the prevalence of *H pylori* infection in different length from the GEJ to the SCJ (*i.e.* between group A and group B). In our study there was no significant difference in the prevalence of *H pylori* infection between group A (41.84%) and group B 48.73%. Furthermore, the prevalence of *H pylori* infection decreased stepwise significantly from RE Grade I (51.72%), grade II (28.57%) to grade III (20.68%) ( $P=0.032$ ). These findings suggest that there is no relationship between gastric *H pylori* infection and GERD, although gastric *H pylori* infection had an apparent protective effect against the progression of GERD.

Barrett's metaplasia is precancerous lesion of esophageal adenocarcinoma and most of adenocarcinoma at the GEJ. Progression from metaplasia, LGD, HGD to invasive cancer

occurs in a stepwise process. It is unquestionably BE, especially LSBE, is linked with gastroesophageal reflux. Our study shows that *H pylori* infection has the protective effect in GERD. But how about BE? The present study demonstrated that there was no significant difference in IM prevalence between group A and group B. Marian *et al* reported that the prevalence of LSBE was higher than that of SSBE and the prevalence of IM is directly proportional to the length of column-lined esophagus<sup>[33]</sup>. This finding, which is different from ours, may explain the reason why the incidence of esophageal adenocarcinoma is on the increase in North America and Europe. However, in our study, the prevalence of SSBE was higher than that of LSBE, which may explain the reason why the incidence of adenocarcinoma at the GEJ is more common than that at the esophagus in China. There was no significant difference in the prevalence of *H pylori* infection between patients with and without IM in both group A and group B. Similarly, there was no significant difference in the prevalence of *H pylori* infection between LSBE and SSBE. However, in distal stomach, the prevalence of *H pylori* infection in patients with IM (56.29%) is much higher than that in those without IM (37.89%) ( $P<0.001$ ), which is in agreement with previous observations<sup>[22,23,43]</sup>. It is suggested that *H pylori* infection is associated with IM in the distal stomach, but may have no protective effect in BE. The distal gastric IM is thought to be less dangerous to progress to gastric carcinoma, and thus it is not suggested to supervise routinely the patients with distal gastric IM<sup>[44]</sup>. However, patients with BE has an overrated risk, about 30 to 125 times to progress to adenocarcinoma than patients without BE<sup>[45, 46]</sup>.

In conclusion, *H pylori* infection may have a protective role in GERD. There is no relationship between gastric *H pylori* infection and BE. *H pylori* infection is associated with IM in the distal stomach.

## REFERENCES

- 1 Devesa SS, Blot WJ, Fraumeni JF Jr. Changing patterns in the incidence of esophageal and gastric carcinoma in the United States. *Cancer* 1998; **83**: 2049-2053
- 2 Blot WJ, Devesa SS, Kneller RW, Fraumeni JF Jr. Rising incidence of adenocarcinoma of the esophagus and gastric cardia. *JAMA* 1991; **265**: 1287-1289
- 3 Zhou Q, Wang LD. Biological characteristics of cardiac cancer. *Huaren Xiaohua Zazhi* 1998; **6**: 636-637
- 4 Ruol A, Parenti A, Zaninotto G, Merigliano S, Costantini M, Cagol M, Alfieri R, Bonavina L, Peracchia A, Ancona E. Intestinal metaplasia is the probable common precursor of adenocarcinoma in barrett esophagus and adenocarcinoma of the gastric cardia. *Cancer* 2000; **88**: 2520-2528
- 5 Rabinovitch PS, Reid BJ, Haggitt RC, Norwood TH, Rubin CE. Progression to cancer in Barrett's esophagus is associated with genomic instability. *Lab Invest* 1989; **60**: 65-71
- 6 Weston AP, Krmpotich PT, Cherian R, Dixon A, Topalovski M. Prospective evaluation of intestinal metaplasia and dysplasia within the cardia of patients with Barrett's esophagus. *Dig Dis Sci* 1997; **42**: 597-602
- 7 Cameron AJ, Lomboy CT, Pera M, Carpenter HA. Adenocarcinoma of the esophagogastric junction and Barrett's esophagus. *Gastroenterology* 1995; **109**: 1541-1546
- 8 Clark GW, Smyrk TC, Burdiles P, Hoeft SF, Peters JH, Kiyabu M, Hinder RA, Bremner CG, DeMeester TR. Is Barrett's metaplasia the source of adenocarcinomas of the cardia? *Arch Surg* 1994; **129**: 609-614
- 9 Warren JR, Marshall BJ. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1983; **1**: 1273-1275
- 10 Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **16**: 1311-1315
- 11 Rauws EA, Langenberg W, Houthoff HJ, Zanen HC, Tytgat GN. Campylobacter pyloridis-associated chronic active antral gastritis. A prospective study of its prevalence and the effects of

- antibacterial and antiulcer treatment. *Gastroenterology* 1988; **94**: 33-40
- 12 **Parsonnet J**, Friedman GD, Vandersteen DP, Chang Y, Vogelstein JH, Orentreich N, Sibley RK. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N Engl J Med* 1991; **325**: 1127-1131
  - 13 **Nomura A**, Stemmermann GN, Chyou PH, Kato I, Perez-Perez GI, Blaser MJ. *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. *N Engl J Med* 1991; **325**: 1132-1136
  - 14 **Isaacson PG**. Gastrointestinal lymphoma. *Hum Pathol* 1994; **25**: 1020-1029
  - 15 **Sigal SH**, Saul SH, Auerbach HE, Raffensperger E, Kant JA, Brooks JJ. Gastric small lymphocytic proliferation with immunoglobulin gene rearrangement in pseudolymphoma versus lymphoma. *Gastroenterology* 1989; **97**: 195-201
  - 16 **Werdmuller BF**, Loffeld RJ. *Helicobacter pylori* infection has no role in the pathogenesis of reflux esophagitis. *Dig Dis Sci* 1997; **42**: 103-105
  - 17 **Koike T**, Ohara S, Sekine H, Iijima K, Kato K, Toyota T, Shimosegawa T. Increased gastric acid secretion after *Helicobacter pylori* eradication may be a factor for developing reflux esophagitis. *Aliment Pharmacol Ther* 2001; **15**: 813-820
  - 18 **Weston AP**, Badr AS, Topalovski M, Cherian R, Dixon A, Hassanein RS. Prospective evaluation of the prevalence of gastric *Helicobacter pylori* infection in patients with GERD, Barrett's esophagus, Barrett's dysplasia, and Barrett's adenocarcinoma. *Am J Gastroenterol* 2000; **95**: 387-394
  - 19 **Walker SJ**, Birch PJ, Stewart M, Stoddard CJ, Hart CA, Day DW. Patterns of colonisation of *Campylobacter pylori* in the oesophagus, stomach and duodenum. *Gut* 1989; **30**: 1334-1338
  - 20 **Newton M**, Bryan R, Burnham WR, Kamm MA. Evaluation of *Helicobacter pylori* in reflux oesophagitis and Barrett's oesophagus. *Gut* 1997; **40**: 9-13
  - 21 **Befrits R**, Granstrom M, Rylander M, Rubio C. *Helicobacter pylori* in 205 consecutive endoscopy patients. *Scand J Infect Dis* 1993; **25**: 189-191
  - 22 **Talley NJ**, Cameron AJ, Shorter RG, Zinsmeister AR, Phillips SF. *Campylobacter pylori* and Barrett's esophagus. *Mayo Clin Proc* 1988; **63**: 1176-1180
  - 23 **Abbas Z**, Hussainy AS, Ibrahim F, Jafri SM, Shaikh H, Khan AH. Barrett's esophagus and *H pylori*. *J Gastroenterol Hepatol* 1995; **10**: 331-333
  - 24 **Genta RM**, Graham DY. Comparison of biopsy sites for the histopathologic diagnosis of *Helicobacter pylori*: a topographic study of *H pylori* density and distribution. *Gastrointest Endosc* 1994; **40**: 342-345
  - 25 **Ei-Zimaity HM**, al-Assi MT, Genta RM, Graham DY. Confirmation of successful therapy of *H pylori* infection: number and sites of biopsies or a rapid urease test. *Am J Gastroenterol* 1995; **90**: 1962-1964
  - 26 **Bayerdorffer E**, Lehn N, Hatz R, Mannes GA, Oertel H, Sauerbruch T, Stolte M. Difference in expression of *Helicobacter pylori* gastritis in antrum and body. *Gastroenterology* 1992; **102**: 1575-1582
  - 27 **Bayerdorffer E**, Oertel H, Lehn N, Kasper G, Mannes GA, Sauerbruch T, Stolte M. Topographic association between active gastritis and *Campylobacter pylori* colonisation. *J Clin Pathol* 1989; **42**: 834-839
  - 28 **Brown KE**, Peura DA. Diagnosis of *Helicobacter pylori* infection. *Gastroenterol Clin North Am* 1993; **22**: 105-115
  - 29 **Skinner DB**, Walther BC, Riddell RH, Schmidt H, Iacone C, DeMeester TR. Barrett's esophagus. Comparison of benign and malignant cases. *Ann Surg* 1983; **198**: 554-565
  - 30 **Spechler SJ**, Zeroogian JM, Antonioli DA, Wang HH, Goyal RK. Prevalence of metaplasia at the gastro-esophageal junction. *Lancet* 1994; **344**: 1533-1536
  - 31 **Weston AP**, Krmpotich P, Makdisi WF, Cherian R, Dixon A, McGregor DH, Banerjee SK. Short segment Barrett's esophagus: clinical and histologic features associated endoscopic findings and association with gastric intestinal metaplasia. *Am J Gastroenterol* 1996; **91**: 981-986
  - 32 **Menke-Pluymers MB**, Hop WC, Dees J, van Blankenstein M, Tilanus HW. Risk factors for the development of an adenocarcinoma in columnar-lined (Barrett) esophagus. The rotterdam esophageal tumor study group. *Cancer* 1993; **72**: 1155-1158
  - 33 **Csendes A**, Maluenda F, Braghetto I, Csendes P, Henriquez A, Quesada MS. Location of the lower oesophageal sphincter and the squamous columnar mucosal junction in 109 healthy controls and 778 patients with different degrees of endoscopic oesophagitis. *Gut* 1993; **34**: 21-27
  - 34 **Tytgat GN**, Hameeteman W, Onstenk R, Schotborg R. The spectrum of columnar-lined esophagus-Barrett's esophagus. *Endoscopy* 1989; **21**: 177-185
  - 35 **Voutilainen M**, Farkkila M, Mecklin JP, Juhola M, Sipponen P. Classical Barrett esophagus contrasted with Barrett-type epithelium at normal-appearing esophagogastric junction. Central finland endoscopy study group. *Scand J Gastroenterol* 2000; **35**: 2-9
  - 36 **Cameron AJ**. Epidemiologic studies and the development of Barrett's esophagus. *Endoscopy* 1993; **25**: 635-636
  - 37 **Hui YP**, Liu Y, Li YH, Ma FC, Wang YM. The relationship between *H pylori* and chronic gastritis. *J Fourth Mil Med Univ* 2001; **22**: 574-575
  - 38 **Graham DY**. *Helicobacter pylori* infection in the pathogenesis of duodenal ulcer and gastric cancer: a model. *Gastroenterology* 1997; **113**: 1983-1991
  - 39 **Graham DY**, Yamaoka Y. *H pylori* and cagA: relationships with gastric cancer, duodenal ulcer, and reflux esophagitis and its complications. *Helicobacter* 1998; **3**: 145-151
  - 40 **Stolte M**, Stadelmann O, Bethke B, Burkard G. Relationships between the degrees of *Helicobacter pylori* colonization and the degree and activity of gastritis, surface epithelial degeneration and mucus secretion. *Z Gastroenterol* 1995; **33**: 89-93
  - 41 **Bercik P**, Verolu E, Armstrong D. Reflux esophagitis and *H pylori*. *Gastroenterology* 1997; **113**: 2020-2021
  - 42 **Labenz J**, Malfertheiner P. *Helicobacter pylori* in gastroesophageal reflux disease: causal agent, independent or protective factor? *Gut* 1997; **41**: 277-280
  - 43 **Spechler SJ**. The role of gastric carditis in metaplasia and neoplasia at the gastroesophageal junction. *Gastroenterology* 1999; **117**: 218-228
  - 44 **Provenzale D**, Kemp JA, Arora S, Wong JB. A guide for surveillance of patients with Barrett's esophagus. *Am J Gastroenterol* 1994; **89**: 670-680
  - 45 **Spechler SJ**, Robbins AH, Rubins HB, Vincent ME, Heeren T, Doos WG, Colton T, Schimmel EM. Adenocarcinoma and Barrett's esophagus: an overrated risk? *Gastroenterology* 1984; **87**: 927-933
  - 46 **Williamson WA**, Ellis FH Jr, Gibb SP, Shahian DM, Aretz HT, Heatley GJ, Watkins E Jr. Barrett's esophagus. Prevalence and incidence of adenocarcinoma. *Arch Intern Med* 1991; **151**: 2212-2216

# Is the vascular endothelial growth factor messenger RNA expression in resectable hepatocellular carcinoma of prognostic value after resection?

Kuo-Shyang Jeng, I-Shyan Sheen, Yi-Ching Wang, Shu-Ling Gu, Chien-Ming Chu, Shou-Chuan Shih, Po-Chuan Wang, Wen-Hsing Chang, Horng-Yuan Wang

**Kuo-Shyang Jeng**, Department of Surgery, Mackay Memorial Hospital, Taipei, Taiwan, China

**I-Shyan Sheen**, Liver Research Unit, Chang Gung Memorial Hospital, Taipei, Taiwan, China

**Yi-Ching Wang, Shu-Ling Gu, Chien-Ming Chu**, Medical Research, Mackay Memorial Hospital, Mackay Junior School of Nursing, Taipei, Taiwan, China

**Shou-Chuan Shih, Po-Chuan Wang, Wen-Hsing Chang, Horng-Yuan Wang**, Department of Internal Medicine, Mackay Memorial Hospital, Taipei, Taiwan, China

**Correspondence to:** I-Shyan Sheen, M.D., Liver Research Unit, Chang Gung Memorial Hospital, No. 199, Tung-Hwa North Road Taipei, Taiwan, China. issheeh.jks@msa.hinet.net

**Telephone:** +886-3-3281200 Ext 8102 **Fax:** +886-2-27065704

**Received:** 2003-10-31 **Accepted:** 2003-12-15

## Abstract

**AIM:** To study whether vascular endothelial growth factor messenger RNA (VEGF mRNA) in the hepatocellular carcinoma (HCC) tissues obtained after curative resection has a prognostic value.

**METHODS:** Using a reverse-transcription polymerase chain reaction (RT-PCR)-based assay, VEGF mRNA was determined prospectively in liver tissues of 50 controls and in HCC tissues of 50 consecutive patients undergoing curative resection for HCC.

**RESULTS:** Among the isoforms of VEGF mRNA, VEGF<sub>165</sub> and VEGF<sub>121</sub> were expressed. By multivariate analysis, a higher level of VEGF<sub>165</sub> in HCC tissue correlated with a significant risk of HCC recurrence ( $P=0.038$ ) and significantly with recurrence-related mortality ( $P=0.045$ ); while VEGF<sub>121</sub> did not. Other significant predictors of HCC recurrence included cellular dedifferentiation ( $P=0.033$ ), an absent or incomplete capsule ( $P=0.020$ ), vascular permeation ( $P=0.018$ ), and daughter nodules ( $P=0.006$ ). The other significant variables of recurrence related mortality consisted of vascular permeation ( $P=0.045$ ), and cellular dedifferentiation ( $P=0.053$ ). The level of VEGF mRNA in HCC tissues, however, did not significantly correlate with tumor size, cellular differentiation, capsule, daughter nodules, vascular permeation, necrosis and hemorrhage of tumors.

**CONCLUSION:** The expression of VEGF mRNA, especially isoform VEGF<sub>165</sub>, in HCC tissues, may play a significant and independent role in the prediction of postoperative recurrence of HCC.

Jeng KS, Sheen IS, Wang YC, Gu SL, Chu CM, Shih SC, Wang PC, Chang WH, Wang HY. Is the vascular endothelial growth factor messenger RNA expression in resectable hepatocellular carcinoma of prognostic value after resection? *World J Gastroenterol* 2004; 10(5): 676-681

<http://www.wjgnet.com/1007-9327/10/676.asp>

## INTRODUCTION

Angiogenesis, the establishment of a neovascular blood supply from preexisting blood vessels, known to be essential for the survival, growth, invasion, and metastasis of tumor cells, is a complex multistep process. The process may include the extracellular matrix remodeling and the binding of angiogenic factors to specific endothelial cell (EC) receptors, leading to EC proliferation, invasion of the basement membrane, migration, differentiation, and formation of new capillary tubes and developing into a vascular network<sup>[1-10]</sup>.

One of the most potent, direct acting, and specific factors with angiogenic activity is vascular endothelial growth factor (VEGF)<sup>[11,12]</sup>.

Hepatocellular carcinoma (HCC), a leading cause of death in Taiwan and many Asian countries, is a highly vascular tumor dependent on neovascularization. Some authors have suggested that VEGF may be a marker for metastasis in HCC because they found markedly elevated VEGF protein levels in HCC patients with remote metastases compared with those without metastasis<sup>[13-15]</sup>. However, most such studies determined VEGF protein concentrations by enzyme immunoassay. To our knowledge, in the prediction of postresection recurrence, little is known about the prognostic significance of VEGF mRNA expression in tumor tissues. We conducted this prospective study to investigate the correlation between VEGF mRNA expression in HCC tissues and postoperative recurrence of HCC.

## MATERIALS AND METHODS

### Study population

Fifty patients (31 men and 19 women, with a mean age of  $56.2 \pm 13.3$  yr) of 58 consecutive patients with HCC undergoing curative hepatectomy from July 2001 to April 2003, were enrolled in this prospective study. Patients who had previously had a hepatectomy or preoperative neoadjuvant ethanol injection or hepatic arterial chemoembolization (TACE) were excluded. Surgical procedures performed included 38 major resections (8 extended right lobectomies, 10 right lobectomies, 8 left lobectomies and 12 two-segmentectomies) and 12 minor resections (10 segmentectomies, 1 subsegmentectomies, and 1 wedge resection). HCC tissues were obtained from all 50 patients after resection. A control group including 10 healthy volunteers without liver disease (5 men, 5 women, mean age 40 yr) and 20 patients with chronic liver disease but without evidence of HCC also received liver biopsy during laparotomy on them for other reasons. All these HCC tissues and liver biopsy tissues (from control group patients) were examined for VEGF mRNA.

After discharge, the patients were assessed regularly to detect tumor recurrence with abdominal ultrasonography (every 2-3 mo during the first 5 yr, then every 4-6 mo thereafter), serum alpha fetoprotein (AFP) and liver biochemistry (every 2 mo during the first 2 yr, then every 4 mo during the following



3 yr, and every 6 mo thereafter), abdominal computed tomography (CT) (every 6 mo during the first 5 yr, then annually), and chest X-ray and bone scans (every 6 mo). Hepatic arteriography was obtained if the other studies suggested possible cancer recurrence. Detection of tumor on any imaging study was defined as clinical recurrence.

Clinicopathological variables analyzed included age, sex (male *vs* female), the presence of liver cirrhosis, Child-Pugh class of liver functional reserve (A *vs* B), hepatitis B virus (HBV) infection (hepatitis B surface antigen), hepatitis C virus (HCV) infection (anti-hepatitis C virus antibody), serum AFP level (<20 ng/mL *vs* 20 to 1 000 ng/mL *vs* >1 000 ng/mL), tumor size (<3 cm *vs* 3 to 10 cm *vs* >10 cm), tumor encapsulation (complete *vs* incomplete or absent), presence of daughter nodules, vascular permeation (including vascular invasion and/or tumor thrombi in either the portal or hepatic vein), and cell differentiation grade (Edmondson and Steiner grades I to IV).

### Detection of VEGF mRNA

It included extraction of RNA, reverse transcription and amplification of cDNA of VEGF and GAPDH by PCR.

### VEGF mRNA of liver tissue

**Extraction of RNA** We homogenized resected tissues completely in 1 mL of RNA-*bee*<sup>TM</sup>, and added 0.2 mL chloroform and shaken vigorously for 15-30 s. We stored the sample on ice for 5 min and centrifuged at 12 000 g for 15 min. We transferred the supernatant to a new 1.5 mL eppendorf tube and precipitated it with 0.5 mL of isopropanol. Precipitation could be as short as 5 min at 4 °C. We centrifuged it at 12 000 g for 5 min at 4 °C. We removed the supernatant and washed the RNA pellet with 1 mL of 750 mL/L ethanol, it dislodged the pellet from the slide of the tube by shaking. We centrifuged at 7 500 g for 5 min at 4 °C and carefully removed ethanol. We removed the supernate and dissolved RNA in DEPC-H<sub>2</sub>O (usually between 50-100 µL) and store at -80 °C.

**Reverse transcription** We heated the RNA sample at 55 °C for 10 minutes and chilled it on ice. We added the following components: (1) 4 µL 5×RT buffer containing 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl<sub>2</sub> and 10 mmol/L DTT(dithiothreitol), (2) 3 µL 10 mmol/L dNTP, (3) 1.6 µL Oligo-d(T)<sub>18</sub> and 0.4 µL random hexamers (N)6 (1 µg/µL), (4) 0.5 uL RNase inhibitor (40 units/µL), (5) 3 µL 25 mmol/L MnCl<sub>2</sub>, (6) 6 µL RNA in DEPC-H<sub>2</sub>O, (7) 0.5 µL DEPC-H<sub>2</sub>O. We incubated it at 70 °C for 2 minutes, chilled it to 23 °C to anneal primers to RNA. We added 1 µL of M-MLV RTase (moloney murine leukemia virus reverse transcriptase, 200 units/µL, Promega). We incubated it for 8 min at 23 °C followed by 60 min at 40 °C. We heated the reaction at 94 °C for 5 min, chilled it on ice and stored cDNA at -20 °C.

**Amplification of cDNA of VEGF and GAPDH by PCR** The sequences of the sense primers were 5' -AGTGTGTGCCCA CTGAGGA-3' (VEGF) and 5' -AGTCAACGGATTTGGT CGTA-3' (GAPDH) and those of the antisense primers were 5' -AGTCAACGGATTTGGTTCGTA-3' (VEGF) and 5' -GGAACATGTAAACCATGTAG-3' (GAPDH). The first polymerase chain reaction (RT-PCR) solution contained 5 µL of the synthesized cDNA solution, 10 µL of 10× polymerase reaction buffer, 500 moi/L each of dCTP, dATP, dGTP and dTTP, 15 pmol of each external primer (EX-sense and EX-antisense), 4 units of Thermus Brockiamus Prozyme DNA polymerase (PROtech Technology Ent. Co., Ltd. Taipei, Taiwan) and water. The PCR cycles were denaturing at 94 °C for 1 min, annealing at 52 °C for 1 min, and primer extension at 72 °C for 1 min. The cycles were repeated 40 times. The PCR product was reamplified with internal primers for nested PCR to obtain a higher sensitivity. The first and second PCR components were the same, but for the primer pairs (IN-sense

and IN-antisense), the final product was electrophoresed on 20g/L agarose gel and stained with ethidium bromide. Four different isoforms of human VEGF were identified, arising from alternative splicing of the primary transcript of a single gene. The majority were VEGF<sub>121</sub> (165 bp) and VEGF<sub>165</sub> (297 bp). The percentage intensity of the VEGF PCR fragment for each liver was relative to a GAPDH PCR fragment (122 bp). The intensity of bands was measured using Fujifilm Science Lab 98 (Image Gauge V3.12). The sensitivity of our assay was assessed using human hepatocytes.

A hepatoblastoma cell line (HepG2) served as a positive control for VEGF mRNA expression. For negative controls, we used EDTA-treated water (filtered and vaporized).

### Statistical analysis

A statistical software (SPSS for Windows, version 8.0, Chicago, Illinois) was employed, with Student's *t*-test used to analyze continuous variables and a chi-square or Fisher's exact test for categorical variables. Parameters relating to the presence of postoperative hAFP mRNA in peripheral blood were analyzed by stepwise logistic regression. A Cox proportional hazards model was used for multivariate stepwise analysis to identify the significant variables for predicting recurrence and mortality. Significance was taken as a *P* value <0.05.

## RESULTS

### RT-PCR analysis of VEGF transcript in liver tissues

VEGF mRNA was expressed in the liver tissues of 10 (VEGF<sub>165</sub> in 10 and VEGF<sub>121</sub> in 6) out of 30 control patients. In the HCC group, isoform VEGF<sub>165</sub> was detected in all the 50 patients (100%) (with a concentration ranging from 0.1860 to 0.7240) and isoform VEGF<sub>121</sub> in 40 patients (80%) (with a concentration ranging from 0.2849 to 1.0298).

We did not detect isoforms VEGF<sub>189</sub> and/or VEGF<sub>206</sub> in either HCC tissues or control liver tissues.

**Table 1** Demographic, clinical and tumor variables of patients with HCC undergoing curative resection (*n*=50)

Variables	No. of patients (%)
Age (mean, years)	56.2±13
Male	31 (62)
Cirrhosis	40 (80)
Child- Pugh's class A	43 (86)
Serum AFP <20 ng/mL	16 (32)
20-10 <sup>3</sup> ng/mL	18 (36)
>10 <sup>3</sup> ng/mL	14 (28)
HBsAg (+)	36 (72)
Anti-HCV (+)	13 (26)
Size of HCC <3 cm	12 (24)
3-10 cm	13 (26)
>10 cm	25 (50)
Edmondson-Steiner's Grade I	4 (8)
Grade II	12 (24)
Grade III	18 (36)
Grade IV	16 (32)
Absent or incomplete capsule	31 (62)
Vascular permeation	29 (58)
Daughter nodules	31 (62)
Tumor necrosis	33 (66)
Tumor hemorrhage	29 (58)

AFP: serum alpha fetoprotein, HBsAg (+): positive hepatitis B surface antigen, Anti-HCV(+): positive hepatitis C virus antibody, Edmondson Steiner grade: differentiation grade.

### Correlation of VEGF mRNA expression and clinical recurrence

During the follow up period (median 1.5 yr, range 1 to 2.5 yr), 16 patients (32%) had clinically detectable recurrence, of whom 7 died. A higher level of isoform VEGF<sub>165</sub> mRNA in HCC tissue correlated significantly with clinical recurrence both univariately ( $P=0.022$ ) and multivariately, ( $P=0.038$ ). Isoform VEGF<sub>121</sub> levels had no such correlation. By multivariate analysis, other significant predictors of recurrence included poor cellular differentiation ( $P=0.033$ ), less encapsulation ( $P=0.020$ ), more vascular permeation ( $P=0.018$ ) and the presence of daughter nodules ( $P=0.006$ ) (Table 2).

**Table 2** Predictors of HCC recurrence

Variable	P values	
	UV	MV
Sex	0.895	-
Age	0.279	-
Size(<3 cm, >10 cm)	0.415	-
Liver cirrhosis	0.510	-
Child-Pugh class	0.528	-
Serum AFP	0.744	-
HBsAg (+)	0.280	-
Anti-HCV (+)	0.481	-
Edmondson Steiner grade	0.0005	0.033
Capsule	<0.0001	0.020
Vascular permeation	<0.0001	0.018
Daughter nodules	<0.0001	0.006
Tumor necrosis	0.344	-
Tumor hemorrhage	0.812	-
Tissue VEGF <sub>165</sub> mRNA	0.022	0.038
Tissue VEGF <sub>121</sub> mRNA	0.622	-

UV: univariate analysis, MV: multivariate analysis, AFP: serum alpha fetoprotein, HBsAg(+): positive hepatitis B surface antigen, Anti-HCV(+): positive hepatitis C virus antibody, Edmondson Steiner grade: differentiation grades I, II vs III, IV, n.s.: not significant.

**Table 3** Correlation between clinical and tumor variables and recurrence-related mortality

Parameters	P values	
	UV	MV
Sex	0.510	-
Age	0.440	-
Size (<3 cm, >10 cm)	0.519	-
Liver cirrhosis	0.510	-
Child-Pugh class	0.548	-
HBsAg (+)	0.351	-
Anti-HCV (+)	0.521	-
Edmondson Steiner grade	<0.001	0.053
Capsule	0.033	n.s.
Vascular permeation	<0.001	0.045
Daughter nodules	0.016	n.s.
Tumor necrosis	0.373	-
Tumor hemorrhage	0.306	-
Tissue VEGF <sub>165</sub> mRNA	0.018	0.045
Tissue VEGF <sub>121</sub> mRNA	0.744	-

UV: univariate analysis, MV: multivariate analysis, AFP: serum alpha fetoprotein, HBsAg (+): positive hepatitis B surface antigen, Anti-HCV(+): positive hepatitis C virus antibody, Edmondson Steiner grade: differentiation grades I, II vs III, IV, n.s.: not significant.

### Correlation of VEGF mRNA expression and recurrence-related death

The level of isoform VEGF<sub>165</sub> in HCC tissue significantly correlated with death due to recurrence both univariately ( $P=0.018$ ) and multivariately ( $P=0.045$ ). By multivariate analysis, a greater degree of vascular permeation significantly correlated with mortality ( $P=0.045$ ), and poor cellular differentiation approached significance ( $P=0.053$ )(Table 3).

### Correlation between VEGF mRNA expression in HCC tissues and clinical and histopathologic features

There was no significant association between isoform of VEGF mRNA and gender, age, serum AFP level, chronic HBV or HCV carriage, tumor size, coexisting cirrhosis, cellular differentiation, capsule, vascular permeation, daughter nodules, tumor necrosis, or tumor hemorrhage ( $P>0.05$ ).

## DISCUSSION

Our study revealed that a higher value of VEGF mRNA isoform <sub>165</sub> in resected HCC tissues was significantly associated with an increased risk of postoperative recurrence and disease mortality. The value of VEGF mRNA isoform <sub>121</sub> in HCC tissues was not significantly predictive of the outcome.

VEGF is also known as a vascular permeability factor and vasculotropin. Its active form is a homodimeric cytokine with molecular weight 34-46 ku. The variation in size due to alternative exon splicing might produce four different isoforms of 121, 165, 189 and 206 amino acids (monomeric size). The last had heparin binding activity<sup>[11,12]</sup>. Different cancers might have different expression of the isoforms. The majority of HCC expressed an abundance of VEGF<sub>121</sub> and VEGF<sub>165</sub><sup>[13-15]</sup>. According to Ferrara's finding, VEGF<sub>165</sub> was the predominantly expressed form in human cDNA libraries as well as in most normal cells and tissues<sup>[12]</sup>.

Some authors have shown that the VEGF level in serum or in tissue is of value for predicting disease progression and prognosis in different cancers, such as the gastrointestinal origins, breast, lung, urothelium, ovary, and lymphoma<sup>[16-26]</sup>. Compared with expression in tumor tissue, the advantage of measurement of serum VEGF level is that it can be performed without tissue specimens and repeated, but it may be influenced by some factors such as coexisting liver cirrhosis, associated infection and platelet activation<sup>[27-32]</sup>.

In addition, the expression of VEGF mRNA in serum might not always correlate significantly with the gene expression level of tumors<sup>[33]</sup>. Therefore, we used liver tissue instead of serum in this study. Warren found VEGF mRNA in hepatocytes and in some Kupffer cells<sup>[34]</sup>. However, release of VEGF mRNA might also be influenced by some cells other than HCC cells<sup>[34]</sup>. The presence of mRNA for VEGF has also been described in T lymphocytes, CD34\* cells, and monocytes<sup>[27, 30]</sup>.

For more accuracy, we chose to measure mRNA expression of VEGF in liver tissue rather than the protein itself. The level of VEGF mRNA did not always correlate with the protein concentration<sup>[32]</sup>. Immunohistochemistry could not distinguish small amounts of protein, which may partly explain the discrepancy in protein and mRNA levels.

The high recurrence rate after resection is the main determinant for the poor outcome of HCC<sup>[35-40]</sup>. Tumor invasiveness variables correlated with recurrence include high serum AFP, hepatitis, vascular permeation, grade of cellular differentiation, infiltration or absence of capsule, tumor size, coexisting cirrhosis, presence of daughter nodules, and multiple lesions. Therefore, a number of studies have been done to see if VEGF correlated with any or all of those factors.

Among reports about the clinical significance of VEGF

expression in HCC, there are considerable discrepancies<sup>[13-15,28,41-48]</sup>. Li found that VEGF mRNA in HCC correlated significantly with portal vein emboli, poorly encapsulated tumors, and microvascular density in HCC tissues<sup>[42]</sup>. Zhou reported that high VEGF expression in HCC was associated with portal vein tumor thrombosis<sup>[43]</sup>. Chow showed that VEGF expression was significantly associated with portal vein tumor thrombosis (sonographic evidence) but not with liver function, tumor volume, gender, severity of liver disease, or tumor grading<sup>[41]</sup>. In addition, the correlation between increased VEGF protein level in HCC and tumor size, number, microscopic venous invasion, metastasis, and recurrence has also been reported.

However, according to our study, a higher expression of VEGF mRNA was significantly correlated with tumor recurrence and recurrence-related mortality but not with the other parameters of tumor invasiveness. VEGF mRNA in HCC tissue thus appears to be an independent risk factor of postoperative recurrence. There are several possible explanations for this dissociation.

The number of study patients is one possible factor. Because most of the reported investigations were performed in small series, the 50 patients we used seemed a more adequate sample size compared with other studies. Another possible explanation for the discrepancies may be the assessment of tumors of different sizes and etiologies.

The relation between tumor size and VEGF mRNA expression might be complex and dynamic because of different vascular growth patterns<sup>[14,48-52]</sup>. If HCCs are about 1.0 cm in diameter, artery-like vessels are not well developed. Capillarization of the blood spaces is present but incomplete, and portal tracts may appear within cancerous nodules. These HCCs are thought to receive a predominantly portal blood supply. As tumor size increases, portal tracts decrease in number, and artery-like vessels gradually increase in number and size. Well-differentiated HCCs measuring 1.0 to 1.5 cm in diameter are in a transitional stage from portal to arterial blood supply, with reduction in portal flow prior to the increase in arterial flow. Therefore, blood flow in HCC at this point would be low and may not be detected on angiography. Hypervascularity becomes easily seen when nodules are larger than 2 cm in diameter. However, with increasing tumor size, VEGF positivity may gradually decrease. According to Yamaguchi, 36.8% of nodules larger than 3.0 cm were VEGF-negative<sup>[48]</sup>. El-Assal showed that, HCCs larger than 5 cm in diameter were less vascular than smaller or medium-sized lesions<sup>[32]</sup>. However, it has been reported that the intercapillary distance increased as the tumor size or weight increased, caused by the significantly different rates of turnover of endothelial cells and neoplastic cells. These complicated changes in vascularity may account for the disparate results among reported studies.

Suzuki reported that VEGF mRNA levels were not correlated with the vascularity of HCCs as seen on angiography<sup>[13]</sup>. On the contrary, Mise *et al* showed that the degree of VEGF mRNA expression was significantly correlated with the intensity of tumor staining in angiograms ( $P < 0.01$ )<sup>[14]</sup>. Because of the complex nature of the angiogenic process, however, it seems that VEGF expression is not the sole contributor to angiogenesis in HCC. Other factors involved in this process may include TGF- $\beta$ , TNF- $\alpha$ , IL-8, *etc.*

The stage of cancer might also influence VEGF expression<sup>[53-55]</sup>. VEGF concentrations have been reported to be significantly higher in advanced rather than early stages of breast, colon and gastric cancer<sup>[16-18,21]</sup>. Chao showed that a lower range of VEGF levels in patients with early-stage HCC overlapped considerably with those of normal controls or patients with chronic hepatitis or cirrhosis<sup>[45]</sup>.

Coexisting liver cirrhosis may influence VEGF expression.

About 80% of our study patients had cirrhosis. Some investigators have found that VEGF expression was significantly higher in cirrhotic liver than in noncirrhotic liver. Furthermore, it has been shown that cirrhosis itself was associated with increased angiogenic activity. According to El-Assal, cirrhotic livers had significantly higher VEGF expressions than noncirrhotic livers<sup>[32]</sup>. In addition, some suggested a possible involvement of VEGF in angiogenesis of cirrhotic liver but not in angiogenesis of HCC<sup>[31,32]</sup>. Akiyoshi suggested that a low serum VEGF level in liver cirrhosis might reflect the degree of liver dysfunction and be associated with the grade of hepatocyte regeneration and VEGF levels decreased with the worsening of Child-Pugh score<sup>[31]</sup>. Whereas, most of our patients belonged to Child-Pugh class A, with resectable lesions, unlike those studied by Akiyoshi.

According to the cell differentiation, the regulation of VEGF may be complex. In our study, VEGF mRNA did not significantly correlate with the grade of cell differentiation. We attribute this to the possibility of different histological grades coexisting in some HCC tissues. Yamaguchi examined VEGF expression immunohistochemically in HCC with various histological grades and sizes<sup>[48]</sup>. In tumors composed of a single histological grade, VEGF expression was the highest in well-differentiated, followed by moderately differentiated, and then poorly differentiated HCC. In tumors consisting of cancerous tissues of two different histological grades, the expression was less intense in the higher-grade HCC component. VEGF was also expressed in the surrounding HCC tissues in which inflammatory cell infiltration was apparent. Based on these findings, VEGF expression in HCC tissues was thought to be partly related to the histological grade, but other cytokines and growth factors could also cooperatively act to enhance or influence VEGF expressions in HCC.

We also found no correlation between VEGF and the absence or presence of fibrous capsule or septum formation, which was in contrast to the findings of Suzuki *et al*<sup>[13]</sup>. The origin of the capsule and fibrous septa in HCC is unclear. Nakashima *et al* suggested the possibility of fibrogenesis at the interface of two tumor nodules with different properties, a process requiring fibrin deposition in the initial stage when the HCC nodule grows to 1.5 cm or larger<sup>[52]</sup>. However, this mechanism has been doubted, since the tumor size did not correlate with the thickness of the capsule or the incidence of its formation.

In our study, a higher level of VEGF mRNA in tumor tissue correlated with more postresection recurrences. We attribute it to two possible mechanisms. One is that the higher angiogenesis may have more invasive nature of cancer to spread into the surrounding tissues. This invasion requires concomitant neovascularization through the sprouting of endothelial cells in extracellular matrix. It has been reported that VEGF could induce both urokinase-type and tissue-type plasmin in endothelial cell, which are the key protease involved in the degradation of the extracellular matrix. The other mechanism is that a shift of VEGF mRNA occurred in liver tissue, which is strongly related to the development of HCC. The progression from preneoplastic to neoplastic tissue would contribute to recurrence.

Surgery remains the potentially curative treatment for patients with HCC. High recurrence rate limits the long term survival. Examination of VEGF mRNA expression in resected HCC tissue may give us information on the risk of postoperative recurrence. Addition of neoadjuvant antiangiogenic therapy after surgery may be considered for such patients. Furthermore, serial measurement of circulating VEGF mRNA during postoperative follow-up to monitor the effect of therapy or the development of recurrence should be further investigated<sup>[56,57]</sup>.

In conclusion, expression of VEGF, especially isoform

VEGF<sub>165</sub>, in HCC tissues may play a significant role in the prediction of postresection recurrence of HCC.

## ACKNOWLEDGEMENT

This study was supported by grants from the Department of Medical Research, Mackay Memorial Hospital, Taiwan (MMH 9237).

## REFERENCES

- Zetter BR.** Angiogenesis and tumor metastasis. *Annu Rev Med* 1998; **49**: 407-424
- Skobe M, Rockwell P, Goldstein N, Vosseler S, Fusenig NE.** Halting angiogenesis suppresses carcinoma cell invasion. *Nat Med* 1997; **3**: 1222-1227
- Marme D.** Tumor angiogenesis: the pivotal role of vascular endothelial growth factor. *World J Urol* 1996; **14**: 166-174
- Folkman J.** Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995; **1**: 27-31
- Folkman J.** Endothelial cells and angiogenic growth factors in cancer growth and metastasis. *Cancer Metastasis Rev* 1990; **9**: 171-174
- Liotta LA, Steeg PS, Stetler-Stevenson WG.** Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 1991; **64**: 327-336
- Fidler IJ, Ellis LM.** The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell* 1994; **79**: 185-188
- Hanahan D, Folkman J.** Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996; **86**: 353-364
- Fox SB, Gatter KC, Harrist AL.** Tumour angiogenesis. *J Pathol* 1996; **179**: 232-237
- Dvorak HF, Brown LF, Detmar M, Dvorak AM.** Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995; **146**: 1029-1039
- Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW.** The vascular endothelial growth factor family: identification of a fourth molecular species and characterization molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol* 1991; **5**: 1806-1814
- Ferrara N, Houck K, Jakeman L, Leung DW.** Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocrinol Rev* 1992; **13**: 18-32
- Suzuki K, Hayashi N, Miyamoto Y, Yamamoto M, Ohkawa K, Ito Y, Sasaki Y, Yamaguchi Y, Nakase H, Noda K, Enomoto N, Arai K, Yamada Y, Yoshihara H, Tujimura T, Kawano K, Yoshikawa K, Kamada T.** Expression of vascular permeability factor/vascular endothelial growth factor in human hepatocellular carcinoma. *Cancer Res* 1996; **56**: 3004-3009
- Mise M, Arai S, Higashitani H, Furutani M, Niwano M, Harada T, Ishigami S, Toda Y, Nakayama H, Fukumoto M, Fujita J, Imamura M.** Clinical significance of vascular endothelial growth factor and basic fibroblast growth factor gene expression in liver tumor. *Hepatology* 1996; **23**: 455-464
- Miura H, Miyazaki T, Kuroda M, Oka T, Machinami R, Kodama T, Shibuya M, Makuuchi M, Yazaki Y, Ohnishi S.** Increased expression of vascular endothelial growth factor in human hepatocellular carcinoma. *J Hepatol* 1997; **27**: 854-861
- Brown LF, Berse B, Jackman RW, Tognazzi K, Guidi AJ, Dvorak HF, Senger DR, Connolly JL, Schnitt SJ.** Expression of vascular endothelial permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. *Hum Pathol* 1995; **26**: 86-91
- Anan K, Morisaki T, Katano M, Ikubo A, Kitsuki H, Uchiyama A, Kuroki S, Tanaka M, Torisu M.** Vascular endothelial growth factor and platelet-derived growth factor are potential angiogenic and metastatic factors in human breast cancer. *Surgery* 1996; **119**: 333-339
- Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM.** Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995; **55**: 3964-3968
- Inoue K, Ozeki Y, Suganuma T, Sugiura Y, Tanaka S.** Vascular endothelial growth factor expression in primary esophageal squamous cell carcinoma: association with angiogenesis and tumor progression. *Cancer* 1997; **79**: 206-213
- Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Senger DR, Dvorak HF.** Expression of vascular permeability factor (vascular endothelial growth factor) and its receptor in adenocarcinomas of the gastrointestinal tract. *Cancer Res* 1993; **53**: 4727-4735
- Maeda K, Chung YS, Ogawa Y, Takatsuka S, Kang SM, Ogawa M, Sawada T, Sowa M.** Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. *Cancer* 1996; **77**: 858-863
- Imoto H, Osaki T, Taga S, Ohgami A, Ichiyoshi Y, Yasumoto K.** Vascular endothelial growth factor expression in non-small-cell lung cancer: prognostic significance in squamous cell carcinoma. *J Thorac Cardiovasc Surg* 1998; **115**: 1007-1014
- Salven P, Ruotsalainen T, Mattson K, Joensuu H.** High pre-treatment serum level of vascular endothelial growth factor (VEGF) is associated with poor outcome in small-cell lung cancer. *Int J Cancer* 1998; **79**: 144-146
- Miyake H, Hara I, Yamanaka K, Gohji K, Arakawa S, Kamidono S.** Elevation of serum level of vascular endothelial growth factor as a new predictor of recurrence and disease progression in patients with superficial urothelial cancer. *Urology* 1999; **53**: 302-307
- Tempfer C, Obrmair A, Hefler L, Haeusler G, Gitsch G, Kainz C.** Vascular endothelial growth factor serum concentrations in ovarian cancer. *Obstet Gynecol* 1998; **92**: 360-363
- Salven P, Teerenhovi L, Joensuu H.** A high pretreatment serum vascular endothelial growth factor concentration is associated with poor outcome in non-Hodgkin's lymphoma. *Blood* 1997; **90**: 3167-3172
- Banks RE, Forbes MA, Kinsey SE, Stanley A, Ingham E, Walters C, Selby PJ.** Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: bearing human hepatocellular carcinoma. *Br J Cancer* 1998; **77**: 956-964
- Jinno K, Tanimizu M, Hyodo I, Nishikawa Y, Hosokawa Y, Doi T, Endo H, Yamashita T, Okada Y.** Circulating vascular endothelial growth factor (VEGF) is a possible tumor marker for metastasis in human hepatocellular carcinoma. *J Gastroenterol* 1998; **33**: 376-382
- Wartiovaara U, Salven P, Mikkola H, Lassila R, Kaukonen J, Joukov V, Orpana A, Ristimäki A, Heikinheimo M, Joensuu H, Alitalo K, Palotie A.** Peripheral blood platelets express VEGF-C and VEGF which are released during platelet activation. *Thromb Haemost* 1998; **80**: 171-175
- Banks RE, Forbes MA, Kinsey SE, Stanley A, Ingham E, Walters C, Selby PJ.** Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: significance for VEGF measurements and cancer biology. *Br J Cancer* 1998; **77**: 956-964
- Akiyoshi F, Sata M, Suzaki H, Uchimura Y, Mitsuyama K, Matsuo K, Tanikawa K.** Serum vascular endothelial growth factor levels in various liver diseases. *Dig Dis Sci* 1998; **43**: 41-45
- El-Assal ON, Yamanai A, Soda Y, Yamaguchi M, Igarashi M, Yamamoto A, Nabika T, Nagasue N.** Clinical significance of microvessel density and vascular endothelial growth factor expression in hepatocellular carcinoma and surrounding liver: possible involvement of vascular endothelial growth factor in the angiogenesis of cirrhotic liver. *Hepatology* 1998; **27**: 1554-1562
- Tokunaga T, Oshika Y, Abe Y, Ozeki Y, Sadehiro S, Kijima H, Tsuchida T, Yamazaki H, Ueyama Y, Tamaoki N, Nakamura M.** Vascular endothelial growth factor (VEGF) mRNA isoform expression pattern is correlated with liver metastasis and poor prognosis in colon cancer. *Br J Cancer* 1998; **77**: 998-1002
- Warren RS, Yuan H, Matli MR, Gillett NA, Ferrara N.** Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest* 1995; **95**: 1789-1797
- Poon RT, Fan ST, Lo CM, Liu CL, Wong J.** Intrahepatic recurrence after curative resection of hepatocellular carcinoma. Long-term results of treatment and prognostic factors. *Ann Surg* 1999; **229**: 216-222
- Jeng KS, Chen BF, Lin HJ.** En bloc resection for extensive hepatocellular carcinoma. Is it advisable? *World J Surg* 1994; **18**: 834-849
- Jeng KS, Sheen IS, Chen BF, Wu JY.** Is the p53 gene mutation of prognostic value in hepatocellular carcinoma after resection? *Arch Surg* 2000; **135**: 1329-1333
- Yamamoto J, Kosuge T, Takayama T, Shimada K, Yamasaki S,**

- Ozaki H, Yamaguchi N, Makuuchi M. Recurrence of hepatocellular carcinoma after surgery. *Br J Surg* 1996; **83**: 1219-1222
- 39 **Jeng JS**, Sheen IS, Tsai YC. Gamma glutamyl transpeptidase messenger RNA may serve as a diagnostic aid in hepatocellular carcinoma. *Br J Surg* 2001; **88**: 986-987
- 40 **Ng IO**, Lai EC, Fan ST, Ng MM, So MK. Prognostic significance of pathologic features of hepatocellular carcinoma. A multivariate analysis of 278 patients. *Cancer* 1995; **76**: 2443-2448
- 41 **Chow NH**, Hsu PI, Lin XZ, Yang HB, Chan SH, Cheng KS, Huang SM, Su JJ. Expression of vascular endothelial growth factor in normal liver and hepatocellular carcinoma: an immunohistochemical study. *Hum Pathol* 1997; **28**: 698-703
- 42 **Li XM**, Tang ZY, Zhou G, Lui YK, Ye SL. Significance of vascular endothelial growth factor mRNA expression in invasion and metastasis of hepatocellular carcinoma. *J Exp Clin Cancer Res* 1998; **17**: 13-17
- 43 **Zhou J**, Tang ZY, Fan J, Wu ZQ, Li XM, Liu YK, Liu F, Sun HC, Ye SL. Expression of platelet-derived endothelial cell growth factor and vascular endothelial growth factor in hepatocellular carcinoma and portal vein tumor thrombus. *J Cancer Res Clin Oncol* 2000; **126**: 57-61
- 44 **Qin LX**, Tang ZY. The prognostic molecular markers in hepatocellular carcinoma. *World J Gastroenterol* 2002; **8**: 385-392
- 45 **Chao Y**, Li CP, Chau GY, Chen CP, King KL, Lui WY, Yen SH, Chang FY, Chan WK, Lee SD. Prognostic significance of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin in patients with resectable hepatocellular carcinoma after surgery. *Ann Surg Oncol* 2003; **10**: 355-362
- 46 **Torimura T**, Sata M, Ueno T, Kin M, Tsuji R, Suzaku K, Hashimoto O, Sugawara H, Tanikawa K. Increased expression of vascular endothelial growth factor is associated with tumor progression in hepatocellular carcinoma. *Hum Pathol* 1998; **29**: 986-991
- 47 **Motoo Y**, Sawabu N, Nakanuma Y. Expression of epidermal growth factor and fibroblast growth factor in human hepatocellular carcinoma: an immunohistochemical study. *Liver* 1991; **11**: 272-277
- 48 **Yamaguchi R**, Yano H, Iemura A, Ogasawara S, Haramaki M, Kojiro M. Expression of vascular endothelial growth factor in human hepatocellular carcinoma. *Hepatology* 1998; **28**: 68-77
- 49 **Yoshiji H**, Kuriyama S, Yoshii J, Yamazaki M, Kikukawa M, Tsujinoue H, Nakatani T, Fukui H. Vascular endothelial growth factor tightly regulates *in vivo* development of murine hepatocellular carcinoma cells. *Hepatology* 1998; **28**: 1489-1496
- 50 **Sakamoto M**, Ino Y, Fujii T, Hirohashi S. Phenotype changes in tumor vessels associated with the progression of hepatocellular carcinoma. *Jpn J Clin Oncol* 1993; **23**: 98-104
- 51 **Terada T**, Nakanuma Y. Arterial elements and perisinusoidal cells in borderline hepatocellular nodules and small hepatocellular carcinomas. *Histopathology* 1995; **27**: 333-339
- 52 **Nakashima O**. Pathological diagnosis of hepatocellular carcinoma. *Nippon Rinsho* 2001; **59**(Suppl 6): 333-341
- 53 **Dirix LY**, Vermeulen PB, Pawinski A, Prove A, Benoy I, De Pooter C, Martin M, Van Oosterom AT. Elevated levels of the angiogenic cytokines basic fibroblast growth factor and vascular endothelial growth factor in sera of cancer patients. *Br J Cancer* 1997; **76**: 238-243
- 54 **Salven P**, Manpaa H, Orpana A, Alitalo K, Joensuu H. Serum vascular endothelial growth factor is often elevated in disseminated cancer. *Clin Cancer Res* 1997; **3**: 647-651
- 55 **Kraft A**, Weindel K, Ochs A, Marth C, Zmija J, Schumacher P, Unger C, Marme D, Gastl G. Vascular endothelial growth factor in the sera and effusions of patients with malignant and nonmalignant disease. *Cancer* 1999; **85**: 178-187
- 56 **Baccala AA**, Zhong H, Clift SM, Nelson WG, Marshall FF, Passe TJ, Gambill NB, Simons JW. Serum vascular endothelial growth factor is a candidate biomarker of metastatic tumor response to *ex vivo* gene therapy of renal cell cancer. *Urology* 1998; **51**: 327-332
- 57 **Denekamp J**. Angiogenesis, neovascular proliferation and vascular pathophysiology as targets for cancer therapy. *Br J Radiol* 1993; **66**: 186-196

Edited by Wang XL Proofread by Zhu LH

# Mechanisms of acupuncture and moxibustion in regulation of epithelial cell apoptosis in rat ulcerative colitis

Huan-Gan Wu, Xiao Gong, Li-Qing Yao, Wei Zhang, Yin Shi, Hui-Rong Liu, Ye-Jing Gong, Li-Bin Zhou, Yi Zhu

**Huan-Gan Wu, Xiao Gong, Wei Zhang, Yin Shi, Hui-Rong Liu, Ye-Jing Gong, Yi Zhu**, Shanghai Institute of Acupuncture-Moxibustion and Meridians, Shanghai 200030, China; Shanghai Research Center of Acupuncture and Meridian, Shanghai 201200, China  
**Li-Qing Yao**, Zhongshan Hospital Fudan University, Shanghai 200032, China

**Li-Bin Zhou**, RuiJin Hospital Shanghai Institute of Endocrinology, Shanghai 200025, China

**Supported by** the National Natural Science Foundation of China, No. 30200368 and Shanghai Commission of Science and Technology, No. 02DZ19150-3 and key program of Shanghai and State Administration of Traditional Chinese Medicine of China

**Correspondence to:** Professor Huan-Gan Wu, Shanghai Institute of Acupuncture-Moxibustion and Meridians, Shanghai 200030, China. wuhuangan@citiz.net

**Telephone:** +86-21-54592009/64383910

**Received:** 2003-07-15 **Accepted:** 2003-08-16

## Abstract

**AIM:** To investigate the effect of acupuncture and moxibustion on epithelial cell apoptosis and expression of Bcl-2, Bax, fas and FasL proteins in rat ulcerative colitis.

**METHODS:** A rat model of ulcerative colitis was established by immunological methods and local stimulation. All rats were randomly divided into model control group (MC), electro-acupuncture group (EA), herbs-partition moxibustion group (HPM). Normal rats were used as normal control group (NC). Epithelial cell apoptosis and expression of Bcl-2, Bax, fas and FasL proteins were detected by TUNEL and immunohistochemical method respectively.

**RESULTS:** The number of epithelial cell apoptosis in MC was significantly higher than that in NC, and was markedly decreased after the treatment with herbs-partition moxibustion or electro-acupuncture. The expression of Bcl-2, Bax, fas and FasL in colonic epithelial cells in MC was higher than that in NC, and was markedly down-regulated by herbs-partition moxibustion or electro-acupuncture treatment.

**CONCLUSION:** The pathogenesis of ulcerative colitis in rats involves abnormality of apoptosis. Acupuncture and moxibustion can regulate the expression of Bcl-2, Bax, fas and FasL proteins and inhibit the apoptosis of epithelial cells of ulcerative colitis in rats by Bcl-2/Bax, fas/FasL pathways.

Wu HG, Gong X, Yao LQ, Zhang W, Shi Y, Liu HR, Gong YJ, Zhou LB, Zhu Y. Mechanisms of acupuncture and moxibustion in regulation of epithelial cell apoptosis in rat ulcerative colitis. *World J Gastroenterol* 2004; 10(5): 682-688  
<http://www.wjgnet.com/1007-9327/10/682.asp>

## INTRODUCTION

Ulcerative colitis (UC) is a non-specific inflammatory intestinal disease. The pathogenesis of ulcerative colitis involves abnormality of apoptosis which is affected by a variety of

factors<sup>[1-3]</sup>. At present, increasing evidence suggests that acceleration of apoptosis of epithelial cells and inhibition of apoptosis of inflammatory cells (such as neutrophil) are closely associated with colonic tissue injury and immunological abnormality in ulcerative colitis.

Apoptosis is determined by the relative expression of serial genes involved in the regulation of apoptosis. Fas/FasL is one of the important pathways of epithelial cell apoptosis in UC. In tissues of UC, the number of FasL positive cells is significantly increased, resulting in apoptosis. FasL expression increases in the focal region of active UC, which directly promotes apoptosis of Fas expressing colonic epithelium. The apoptosis promoting gene bax also plays an important role in apoptosis. The ratio of bax/bcl-2 determines whether apoptosis occurs or not. Excessive expression of bax promotes apoptosis.

In the present study, a rat model of UC was established by immunological method and local stimulation. After the treatment with electro-acupuncture and herbs-partition moxibustion, the number of colonic epithelial cell apoptosis and the expression of Bcl-2/Bax and Fas/FasL proteins were detected by TUNEL and immunohistochemistry respectively for elucidating the mechanism of acupuncture and moxibustion underlying colonic epithelial cell apoptosis in rat UC.

## MATERIALS AND METHODS

### Experimental animals and materials

Two hundred male SD rats (weighting 200±20 g) were provided by Experimental Animal Center of Shanghai University of TCM. TUNEL kits was purchased from Boehringer Mannheim (Germany). Bax, Bcl-2 and FasL kits were from Dako (Denmark). Fas was from Santa-cruz (USA).

### Methods

**Animal model and therapeutic methods** Establishment of animal model: According to Experimental Methodology of Pharmacology<sup>[4]</sup>, UC rat model was established by immunological method and local stimulation. Colonic mucosa was prepared from human fresh surgical colonic specimens, homogenized by adding appropriate amount of normal saline and centrifuged for 30 min at 3 000 r/min. The supernatant was removed for the measurement of protein concentration and then mixed with Freund adjuvant. The antigen fluid was first injected into the plantar pedis of the model group rats, then into the plantar pedis, dorsum, inguen and abdominal cavity (no Freund adjuvant in the last injection) on the tenth, seventeenth, twenty-fourth and thirty-first day respectively. When a certain titer of serum anti-colonic antibody was reached, 3 mL 3% formalin and 2 mL antigen fluid (no Freund adjuvant) were administered by enema successively. The rats in NC were administrated with normal saline as the same procedure of MC.

**Treatment:** After the ulcerative colitis rat model was built, the animals were randomly divided into model control group (MC 8), electro-acupuncture group (EA 8), herbs-partition moxibustion group (HPM 8) and normal control group (NC 6). HPM: Moxa cones made of refined mugwort floss were placed on the medicinal formula (medicinal formula dispensing: *Radix Aconiti praeparata*, *cortex Cinnamomi*, et al) for Qihai (RN



6) and Tianshu (ST 25, bilateral) and ignited. Two moxa cones were used for each treatment once a day and 14 times as a course. EA: Tianshu (bilateral) and Qihai were acupunctured and then stimulated by intermittent pulse with 2HZ frequency, 4 mA intensity for 20 minutes once a day and 14 times as a course.

After treatment, four group rats were killed simultaneously. The distal 6 cm long colons were dissected and reserved in formaldehyde solution.

**TUNEL analysis** Formalin fixed specimens were embedded in paraffin using standard procedures. Deparaffinised and rehydrated sections were immersed in 3mL/L  $H_2O_2$  for 30 min at room temperature and digested with proteinase K for 20 min at 37 °C. The sections were immersed in 1g/L Triton-100 and then incubated with TUNEL mixture for 1 hour at room temperature, with streptavidin-HRP(1:400) for 30 min at 37 °C. The sections were stained with 0.4g/L DAB and treated with 3mL/L  $H_2O_2$  for 10 min and with hematoxylin for 1 min. The results were observed with light microscopy. Positive reaction was shown by brown color. The apoptotic cells were counted as the mean of cells in 3 visual fields of one section. The data were analysed by *q* test, using statistical package SPSS.

**Immunohistochemistry** Formalin fixed specimens were embedded in paraffin using standard procedures. Sections attached on carry sheet glass were autoclaved at 58 °C for 24 h. Deparaffinised and rehydrated sections were immersed in 10mL/L  $H_2O_2$  for 20 min and washed three times, each time for 3 min with PBS. Sections were preincubated with 10mL/L normal goat anti rabbit serum for 20 min at room temperature and then incubated with the first antibodies diluted for 18 h at 4 °C and Envision reagent for 30 min at 37 °C. Sections were stained by 0.4g/L DAB with 0.3mL/L  $H_2O_2$  for 8 min and hematoxylin for 30 s. The results were observed under light microscope.

Positive specimens were used as positive controls. The result of PBS instead of the first antibody was used as negative control. The positive reactions showed brown particles. The positive cells expressing Bcl-2, Bax, fas and FasL were counted as the mean of cells in 3 visual fields of one section. The data were analysed by *q* test, using statistical package SPSS.

## RESULTS

The effect of acupuncture and moxibustion on epithelial cell apoptosis in UC rats is shown in Table 1 and Figure 1(A-D).

**Table 1** Results of epithelial cell apoptosis in different groups

Group	<i>n</i>	Number of apoptotic cells (mean±SD)
NC	6	20.61±1.99
MC	8	66.21±8.51 <sup>b</sup>
HPM	8	33.58±3.59 <sup>bd</sup>
EA	8	34.29±2.70 <sup>bd</sup>

<sup>b</sup>*P*<0.01 vs NC; <sup>d</sup>*P*<0.01 vs MC.

Table 1 shows that the number of epithelial cell apoptosis in MC was significantly increased compared with that of NC (*P*<0.01). The number of epithelial cell apoptosis in EA and HPM was remarkably decreased compared with that of MC (*P*<0.01), but was not as low as that of NC (*P*<0.01).

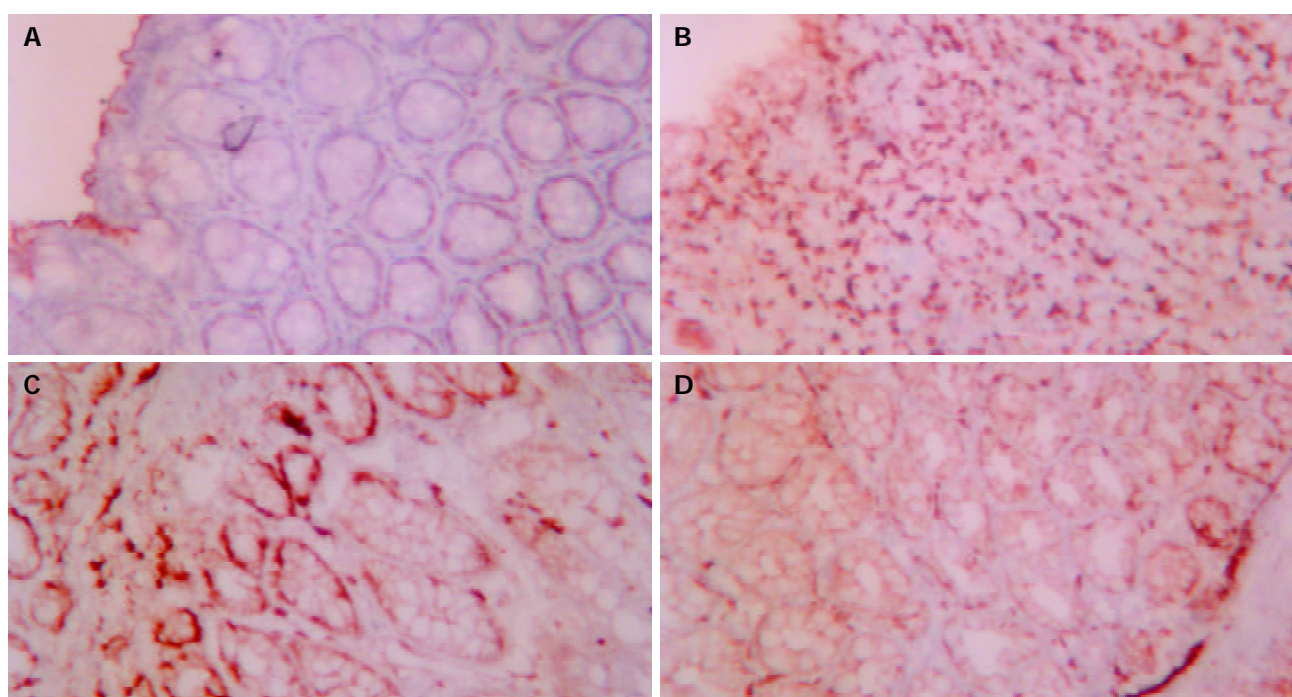
*The effect of acupuncture and moxibustion on Bax expression in colonic epithelia of UC rats is shown in Table 2 and Figure 2(A-D)*

**Table 2** Bax expression in colonic epithelia of different groups (mean±SD)

Group	<i>n</i>	Area of expression	Intensity of expression
NC	6	35 905.06±2 987.97	0.1683±0.0105
MC	8	52 451.13±3 174.10 <sup>b</sup>	0.2558±0.0142 <sup>b</sup>
HPM	8	39 561.50±1 382.94 <sup>d</sup>	0.1900±0.0047 <sup>d</sup>
EA	8	38 477.79±3 309.19 <sup>d</sup>	0.1796±0.0117 <sup>d</sup>

<sup>b</sup>*P*<0.01 vs NC; <sup>d</sup>*P*<0.01 vs MC.

Table 2 shows that the area and intensity of Bax expression in the colonic epithelia of MC were significantly increased compared with that of NC (*P*<0.01). The area and intensity



**Figure 1** A: Epithelial cell apoptosis in NC ×200, B: Epithelial cell apoptosis in MC ×200, C: Epithelial cell apoptosis in EA ×200, D: Epithelial cell apoptosis in HPM ×200.

of Bax expression in the colonic epithelia of PHM and EA were markedly decreased compared with that of MC, but showed no significant difference when compared with that of NC.

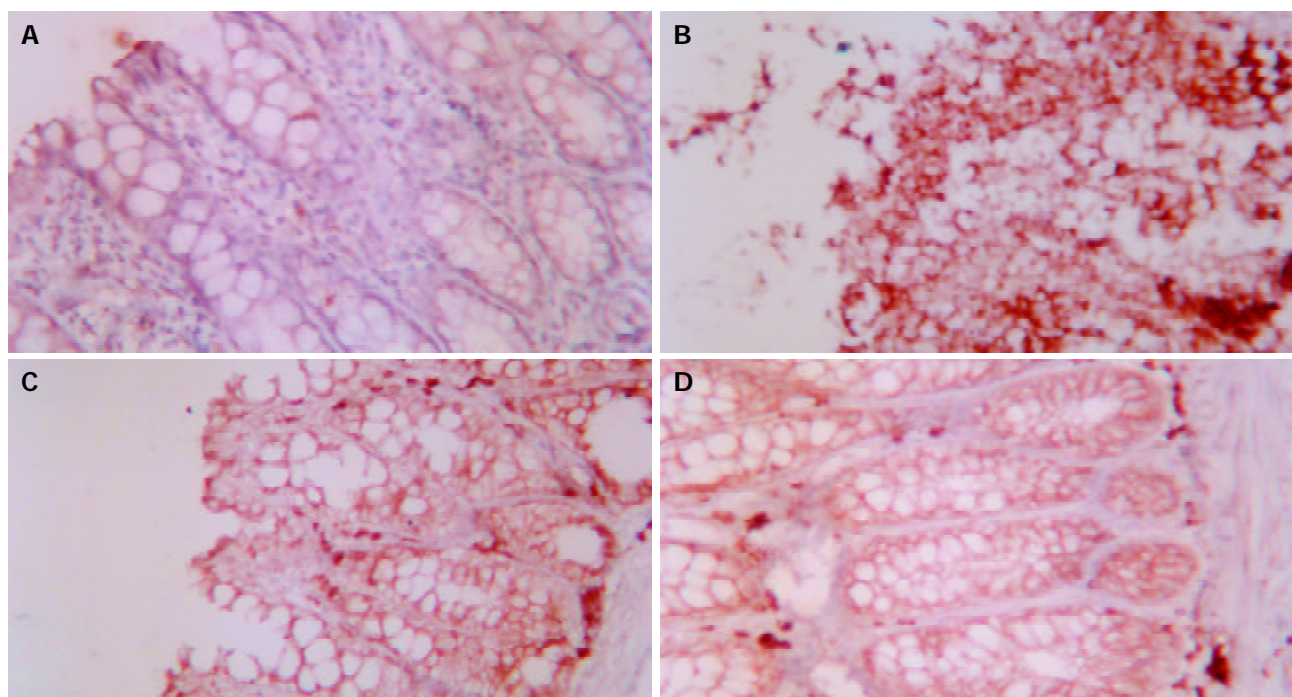
***The effect of acupuncture and moxibustion on Bcl-2 expression in the colonic epithelium of UC rats is shown in Table 3 and Figure 3(A-D)***

Table 3 shows that the area and intensity of Bcl-2 expression in the colonic epithelia of MC were significantly increased compared with that of NC ( $P<0.01$ ). The area and intensity of Bcl-2 expression in the colonic epithelia of PHM and EA were

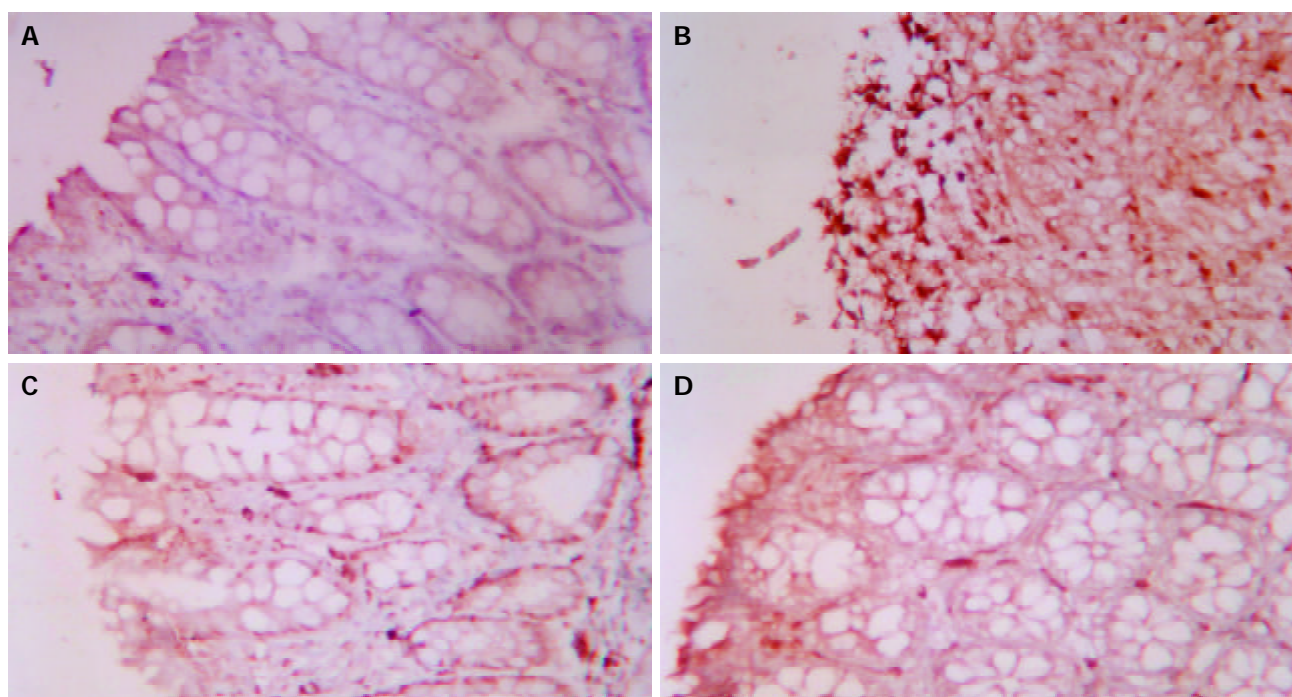
markedly decreased compared with that of MC, but which were not as low as that of NC.

***The effect of acupuncture and moxibustion on Fas expression in the colonic epithelium of UC rats is shown in Table 4 and Figure 4(A-D)***

Table 4 shows that the area and intensity of Fas expression in the colonic epithelia of MC were significantly increased compared with that of NC ( $P<0.01$ ). The area and intensity of Fas expression in the colonic epithelia of PHM and EA were markedly decreased compared with that of MC ( $P<0.01$ ), but still had a significant difference compared with that of NC ( $P<0.05$ ).



**Figure 2** A: Bax expression in colonic epithelia of NC  $\times 200$ , B: Bax expression in colonic epithelia of MC  $\times 200$ , C: Bax expression in colonic epithelia of EA  $\times 200$ , D: Bax expression in colonic epithelia of HPM  $\times 200$ .



**Figure 3** A: Bcl-2 expression in colonic epithelia of NC  $\times 200$ , B: Bcl-2 expression in colonic epithelia of MC  $\times 200$ , C: Bcl-2 expression in colonic epithelia of EA  $\times 200$ , D: Bcl-2 expression in colonic epithelia of HPM  $\times 200$ .

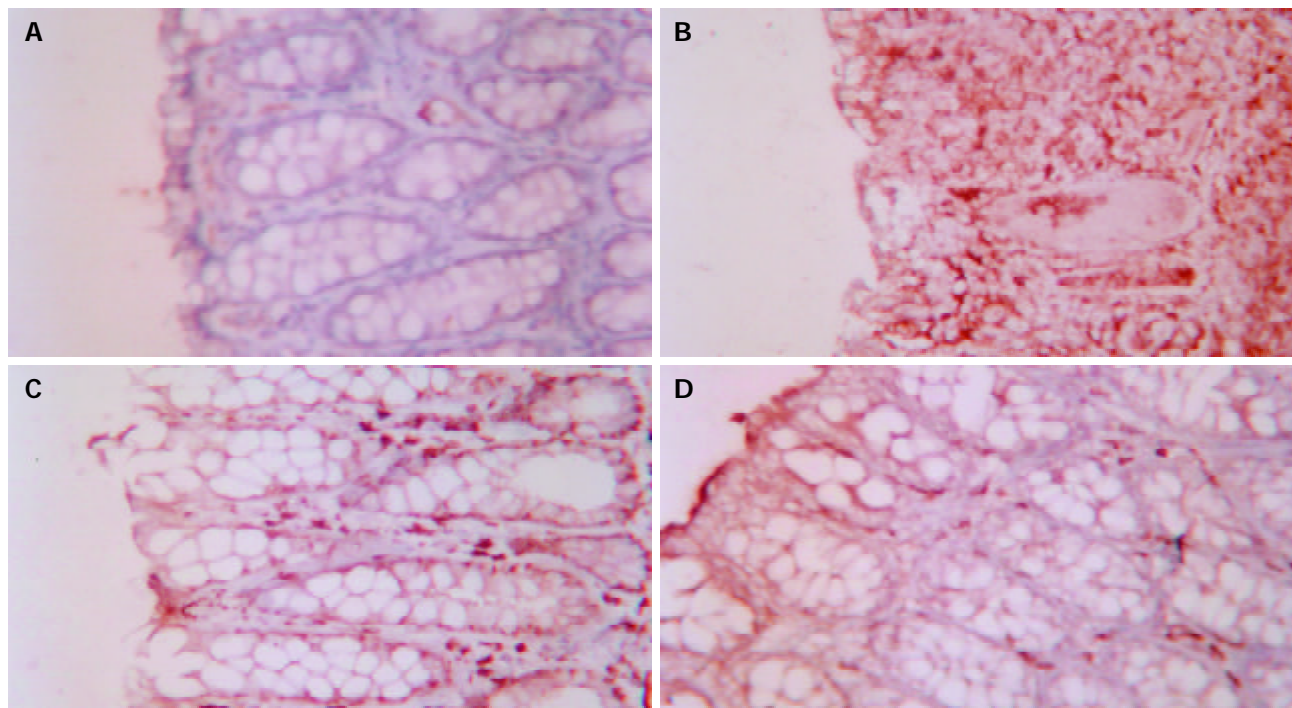
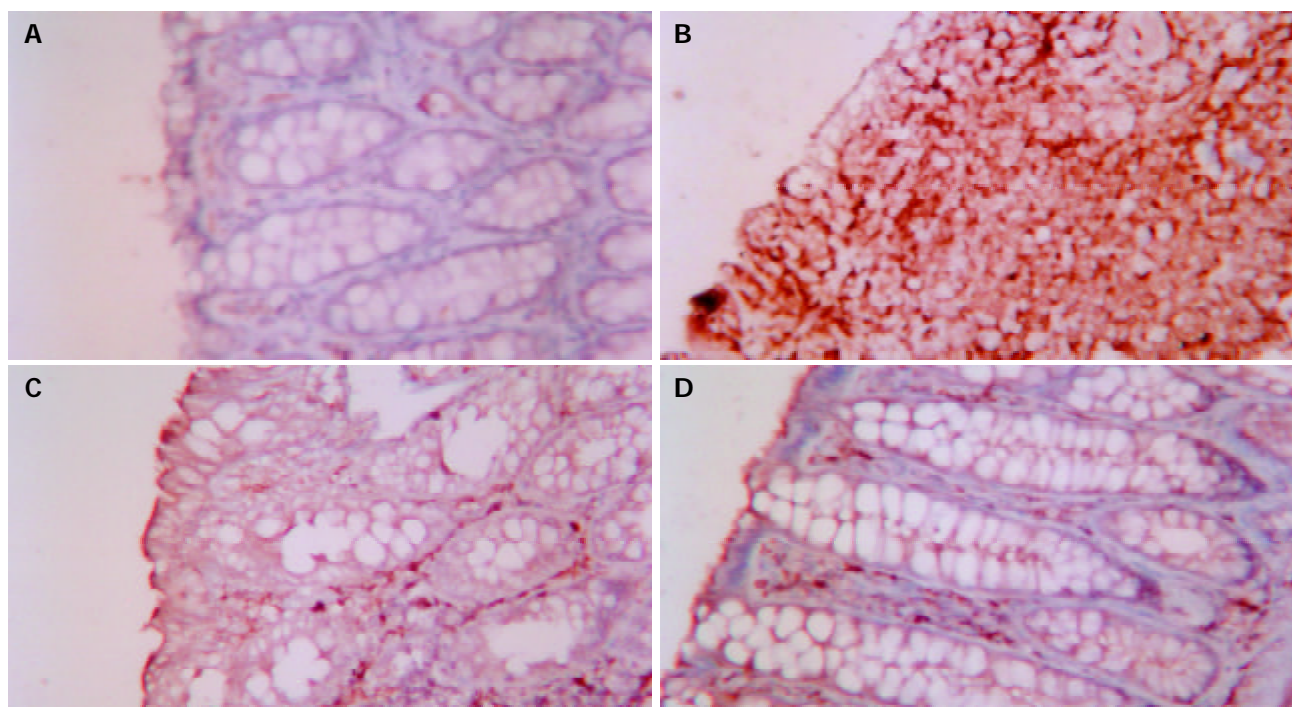


**Table 3** Bcl-2 expression in colonic epithelia of different groups (mean±SD)

Group	n	Area of expression (μm <sup>2</sup> )	Intensity of expression
NC	6	30 863.61±2 273.44	0.1539±0.0114
MC	8	44 757.67±28.1.53 <sup>b</sup>	0.2242±0.0196 <sup>b</sup>
HPM	8	40 061.63±4 937.84 <sup>be</sup>	0.1979±0.0177 <sup>bd</sup>
EA	8	39 219.04±3 449.84 <sup>db</sup>	0.1875±0.0133 <sup>bd</sup>

<sup>b</sup>*P*<0.01 vs NC; <sup>d</sup>*P*<0.01 vs MC; <sup>e</sup>*P*<0.05 vs MC.**Table 4** Fas expression in colonic epithelia of different groups (mean±SD)

Group	n	Area of expression (μm <sup>2</sup> )	Intensity of expression
NC	6	33 764.67±4 422.37	0.1722±0.0153
MC	8	50 262.08±4 780.34 <sup>b</sup>	0.2500±0.0212 <sup>b</sup>
HPM	8	37 992.29±3 239.23 <sup>de</sup>	0.1825±0.0200 <sup>d</sup>
EA	8	38 913.21±4 669.80 <sup>de</sup>	0.1850±0.0138 <sup>d</sup>

<sup>b</sup>*P*<0.01 vs NC; <sup>d</sup>*P*<0.01 vs MC; <sup>e</sup>*P*<0.05 vs NC.**Figure 4** A: Fas expression in colonic epithelia of NC ×200, B: Fas expression in colonic epithelia of MC ×200, C: Fas expression in colonic epithelia of EA ×200, D: Fas expression in colonic epithelia of HPM ×200.**Figure 5** A: FasL expression in colonic epithelia of NC ×200, B: FasL expression in colonic epithelia of MC ×200, 5: FasL expression in colonic epithelia of EA ×200, D: FasL expression in colonic epithelia of HPM ×200.

**The effect of acupuncture and moxibustion on FasL expression in the colonic epithelium of UC rats is shown in Table 5 and Figure 5(A-D)**

**Table 5** FasL expression in colonic epithelia of different groups (mean±SD)

Group	n	Area of expression (μm <sup>2</sup> )	Intensity of expression
NC	6	33 063.56±3 347.24	0.1561±0.0080
MC	8	44 566.58±4 637.23 <sup>b</sup>	0.2600±0.0105 <sup>b</sup>
HPM	8	38 825.58±2 495.51 <sup>bd</sup>	0.1838±0.0156 <sup>bd</sup>
EA	8	38 553.29±3 489.38 <sup>bd</sup>	0.1850±0.0108 <sup>bd</sup>

<sup>b</sup>P<0.01 vs NC; <sup>d</sup>P<0.01 vs MC.

Table 5 shows that the area and intensity of FasL expression in the colonic epithelia of MC were significantly increased compared with that of NC ( $P<0.01$ ). The area and intensity of FasL expression in the colonic epithelia of PHM and EA were markedly decreased compared with that of MC ( $P<0.01$ ), which had a significant difference compared with that of NC ( $P<0.01$ ).

## DISCUSSION

UC is a non-specific inflammatory intestinal disease. The incidence of UC in our country has an increasing trend yearly. The pathogenesis of ulcerative colitis in rats involved in the abnormality of apoptosis<sup>[5-8]</sup>. Increasing evidence showed that acceleration of epithelial cell apoptosis and inhibition of inflammatory cell apoptosis were closely associated with colonic tissue injury and immunological abnormality in ulcerative colitis<sup>[9-11]</sup>.

Studies showed that cell proliferation, differentiation and apoptosis of epithelial cells in intestines mucosa were a dynamic equilibrium process, and neonate epithelial cells migration along crypt villi from pit cells became mature villous epithelial cells. In physiological condition, apoptosis only occurred on superficial epithelial cells of intestine. In pathologic status, this sequence was destructive. For example, at the area of active inflammation, the apoptotic rate of neonate epithelial cells is accelerated and pit cells were superproliferative<sup>[12-14]</sup>. This alteration would lead to destruction of epithelial barrier of colon and imbalance of intestinal function of absorption and excretion.

Apoptosis is a procedure of death adjusted by a flock of apoptotic genes, the cell apoptosis was determined by the relative gene expression level of a series of apoptosis genes<sup>[15-17]</sup>. The bcl-2 gene kindred is an important apoptosis adjusting gene, the position of Bcl-2 protein is at mitochondrial membrane, endoplasmic reticulum and nuclear membrane. As it could prolong the life of cells, it has been generally accepted as an antiapoptosis gene<sup>[18-23]</sup>. Bax is a new member of bcl-2 gene kindred, it could form a dimer with bcl-2 to inhibit its function<sup>[24-27]</sup>. The relative expression ratio of bcl-2 and bax determines whether apoptosis happens in cells or not. When expression of bax gained advantage, apoptosis would occur and when the expression of bcl-2 gained advantage, the cells would continue to exist<sup>[28-32]</sup>. Many studies have shown that abnormal apoptosis in ulcerative colitis could be affected by many agents<sup>[33-36]</sup>.

This study showed that persistent inflammation resulted in the abnormal increase of epithelial cell apoptosis in UC rats. Meanwhile, the area and intensity of Bcl-2 and Bax expression in colonic epithelia of MC were significantly increased compared with that of NC, suggesting that epithelial cell apoptosis is abnormally active. The upregulation of Bcl-2 and Bax expression in colonic epithelia increased the number of

apoptotic cells, which might be one of the important mechanisms of colonic pathological changes in UC. After the treatment with electro-acupuncture and herbs-partition moxibustion, the ulceration of colonic tissues in both groups was markedly improved and the tissue structure was well restored. The number of apoptotic cells in colonic tissues in EA and HPM was significantly decreased compared with that of NC. The area and intensity of Bcl-2 and Bax expression in colonic epithelia of PHM and EA were markedly decreased compared with that of MC. Especially, Bax expression was much downregulated. The above results showed that electro-acupuncture and herbs-partition moxibustion could inhibit colonic epithelial cell apoptosis of UC rats by decreasing Bcl-2 and Bax expression. The extent of Bcl-2 and Bax expression in colonic epithelia of UC rats downregulated by acupuncture and herbs-partition moxibustion was different, thus the relative ratio of Bcl-2 and Bax expression in colonic epithelia was changed due to the inhibition of the abnormal increase of epithelial cell apoptosis in UC. The above results showed that down-regulation of the inflammatory reaction of colonic epithelia in UC rats, inhibition of the injurious effect of a variety of proinflammatory cytokines on colonic tissues, decrease of Bcl-2 and Bax expression of epithelial cells and regulation of the relative ratio of Bcl-2 and Bax expression could change the active state of colonic epithelial cell apoptosis due to its decrease. This is one of the important mechanisms of acupuncture and moxibustion in regulating apoptosis and treating UC.

Many studies<sup>[37-39]</sup> have shown Fas/FasL is an important pathway of epithelial cell apoptosis in UC. Fas is also named Apo-1 or CD95, belongs to tumour necrosis factor receptor (TNFR) kindred, it has comprehensive expressions in various histocytes and can bind anti-Fas antibody or FasL to change the constitution of cell surface. So it can transmit signals in cells to switch on apoptosis mechanism, leading to apoptosis of cells that express Fas. The function of Fas/FasL is to maintain immune stability of body and balance of body's apoptosis<sup>[40-44]</sup>. Normally colonic epithelium could express Fas, and a small quantity of cells could express FasL in the site where the number of apoptosis cells was markedly increased. When UC occurred, because of stimulation by inflammation, the increase of FasL expression would cause apoptosis<sup>[45,46]</sup>. The high expression of FasL in active UC could cause apoptosis of cells expressing Fas<sup>[47-49]</sup>, this would accelerate migration and activity of neutrophils and lymphocytes, causing progressive mucosal lesion of UC<sup>[50-52]</sup>. Therefore, the study of epithelial cell apoptosis may develop a new effective therapeutic approach to UC.

The study showed that the area and intensity of Fas and FasL expression in colonic epithelia of MC were significantly increased compared with that of NC as apoptosis increased, suggesting that the high expression of Fas and FasL plays an important role in epithelial cell apoptosis in UC. After the treatment with electro-acupuncture and herbs-partition moxibustion, Fas and FasL expressions in colonic epithelia of both groups were markedly downregulated compared with MC, and the number of apoptosis cells was also decreased. The above results showed that regulating the abnormal expression of Fas and FasL in colonic tissues of UC rats and decreasing its epithelial cell apoptosis might be one of the important mechanisms of acupuncture and moxibustion in treating UC. Our previous study showed that acupuncture and moxibustion could markedly inhibit the expression of proinflammatory cytokines such as IL-1β and IL-6<sup>[53-56]</sup>. It is suggested that acupuncture and moxibustion can regulate Fas and FasL expression in colonic tissues of UC rats, and may be associated with the inhibition of the activation of macrophages in colonic epithelia and decrease of the expression of proinflammatory cytokines such as IL-1β and IL-6. Further activation of

macrophages in the blocked initial cascade reaction of inflammation and immunity in colonic epithelia can be effectively controlled. Stimulation of inflammatory cytokines on colonic tissues is relieved and stability of immunological function in UC rats is restored.

## REFERENCES

- Xia B**, Shivananda S, Zhang GS, Yi JY, Crusius JBA, Peña AS. Inflammatory bowel disease in Hubei Province of China. *China Natl J New Gastroenterol* 1997; **3**: 119-120
- Xia B**, Guo HJ, Crusius JBA, Deng CS, Meuwissen SGM, Peña AS. *In vitro* production of TNF- $\alpha$ , IL-6 and sIL-2R in Chinese patients with ulcerative colitis. *World J Gastroenterol* 1998; **4**: 252-255
- Hu QY**, Hu XY, Jiang Y. Clinical investigation of ulcerative colitis patients treated by integrated traditional Chinese and Western medicine. *World J Gastroenterol* 1998; **4**(Suppl 2): 93-94
- Xu SY**, Bian RL, Chen X. Experimental methodology of pharmacology. Beijing: People's Health Publishing House 1982: 892
- Seidelin JB**, Nielsen OH. Apoptosis in chronic inflammatory bowel disease. The importance for pathogenesis and treatment. *Ugeskr Laeger* 2003; **165**: 790-792
- Sasaki S**, Yoneyama H, Suzuki K, Suriki H, Aiba T, Watanabe S, Kawauchi Y, Kawachi H, Shimizu F, Matsushima K, Asakura H, Narumi S. Blockade of CXCL10 protects mice from acute colitis and enhances crypt cell survival. *Eur J Immunol* 2002; **32**: 3197-3205
- Vetuschi A**, Latella G, Sferra R, Caprilli R, Gaudio E. Increased proliferation and apoptosis of colonic epithelial cells in dextran sulfate sodium-induced colitis in rats. *Dig Dis Sci* 2002; **47**: 1447-1457
- Sipos F**, Molnar B, Zagoni T, Tulassay Z. Changes in cell kinetics and clinical course in inflammatory bowel diseases. *Orv Hetil* 2002; **143**: 1175-1181
- Buttke TM**, Sandstrom PA. Oxidative stress as a mediator of apoptosis. *Immunol Today* 1994; **15**: 7-10
- Arai N**, Mitomi H, Ohtani Y, Igarashi M, Kakita A, Okayasu I. Enhanced epithelial cell turnover associated with p53 accumulation and high p21WAF/CIP1 expression in ulcerative colitis. *Mod Pathol* 1999; **12**: 604-611
- Bu P**, Keshavarzian A, Stone DD, Liu J, Le PT, Fisher S, Qiao L. Apoptosis: one of the mechanisms that maintains unresponsiveness of the intestinal mucosal immune system. *J Immunol* 2001; **166**: 6399-6403
- Sasaki S**, Yoneyama H, Suzuki K, Suriki H, Aiba T, Watanabe S, Kawauchi Y, Kawachi H, Shimizu F, Matsushima K, Asakura H, Narumi S. Blockade of CXCL10 protects mice from acute colitis and enhances crypt cell survival. *Eur J Immunol* 2002; **32**: 3197-3205
- Dieckgraefe BK**, Crimmins DL, Landt V, Houchen C, Anant S, Porche-Sorbet R, Ladenson JH. Expression of the regenerating gene family in inflammatory bowel disease mucosa: Reg Ialpha upregulation, processing, and anti apoptotic activity. *J Invest Med* 2002; **50**: 421-434
- Ruemmele FM**, Seidman EG. Cytokine-intestinal epithelial cell interactions: implications for immune mediated bowel disorders. *Zhonghua Minguo Xiaoe Keyi Xuehui Zazhi* 1998; **39**: 1-8
- Huang XM**. Bcl-2 with its protein and regulation of apoptosis. *Foreign Med Sci Sec Mol Biol* 1997; **19**: 16-19
- Wu K**, Zhao Y. Investigation progress of apoptosis. *Foreign Med Sci Sec Mol Biol* 2001; **24**: 134-136
- Mercer WE**, Shields MT, Lin D, Appella E, Ullrich SJ. Growth suppression induced by wild-type p53 protein is accompanied by selective down-regulation of proliferating cell nuclear antigen expression. *Proc Natl Acad Sci U S A* 1991; **88**: 1958-1962
- Ohman L**, Franzen L, Rudolph U, Birnbaumer L, Hornquist EH. Regression of Peyer's patches in G alpha i2 deficient mice prior to colitis is associated with reduced expression of Bcl-2 and increased apoptosis. *Gut* 2002; **51**: 392-397
- Yan J**, Ouyang Q, Liu WP, Li GD, Li FY. Apoptosis and proliferation of epithelial cells in ulcerative colitis. *Zhonghua Xiaohua Neijing Zazhi* 2001; **18**: 161-163
- Jiang XL**, Quan QZ, Sun ZQ, Wang YJ, Qi F, Wang D, Zhang XL. The expression of apoptosis adjust protein of ulcerative colitis patient's lymphocyte. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 903-904
- Mayer B**, Oberbauer R. Mitochondrial regulation of apoptosis. *News Physiol Sci* 2003; **18**: 89-94
- Buduneli E**, Genel F, Atilla G, Kutukculer N. Evaluation of p53, bcl-2, and interleukin-15 levels in gingival crevicular fluid of cyclosporin A-treated patients. *J Periodontol* 2003; **74**: 506-511
- Sohn SK**, Jung JT, Kim DH, Kim JG, Kwak EK, Park T, Shin DG, Sohn KR, Lee KB. Prognostic significance of bcl-2, bax, and p53 expression in diffuse large B-cell lymphoma. *Am J Hematol* 2003; **73**: 101-107
- Chiu CT**, Yeh TS, Hsu JC, Chen MF. Expression of Bcl-2 family modulated through p53-dependent pathway in human hepatocellular carcinoma. *Dig Dis Sci* 2003; **48**: 670-676
- Korkolopoulou P**, Lazaris ACH, Konstantinidou AE, Kavantzias N, Patsouris E, Christodoulou P, Thomas-Tsagli E, Davaris P. Differential expression of bcl-2 family proteins in bladder carcinomas. Relationship with apoptotic rate and survival. *Eur Urol* 2002; **41**: 274-283
- Scorrano L**, Korsmeyer SJ. Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. *Biochem Biophys Res Commun* 2003; **304**: 437-444
- Oltvai ZN**, Millman CL, Korsmeyer SJ. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 1993; **74**: 609-619
- Korkolopoulou P**, Oates J, Kittas C, Crocker J. p53, c-myc, p62 and proliferating cell nuclear antigen (PCNA) expression in non-Hodgkin's lymphomas. *J Clin Pathol* 1994; **47**: 9-14
- Iimura M**, Nakamura T, Shinozaki S, Iizuka B, Inoue Y, Suzuki S, Hayashi N. Bax is downregulated in inflamed colonic mucosa of ulcerative colitis. *Gut* 2000; **47**: 228-235
- Mueller E**, Vieth M, Stolte M, Mueller J. The differentiation of true adenomas from colitis-associated dysplasia in ulcerative colitis: a comparative immunohistochemical study. *Hum Pathol* 1999; **30**: 898-905
- Ina K**, Itoh J, Fukushima K, Kusugami K, Yamaguchi T, Kyokane K, Imada A, Binion DG, Musso A, West GA, Dobrea GM, McCormick TS, Lapetina EG, Levine AD, Ottaway CA, Fiocchi C. Resistance of Crohn's disease T cells to multiple apoptotic signals is associated with a Bcl-2/Bax mucosal imbalance. *J Immunol* 1999; **163**: 1081-1090
- Kraus MD**, Shahsafaei A, Antin J, Odze RD. Relationship of Bcl-2 expression with apoptosis and proliferation in colonic graft versus host disease. *Hum Pathol* 1998; **29**: 869-875
- Ilyas M**, Tomlinson IP, Hanby AM, Yao T, Bodmer WF, Talbot IC. Bcl-2 expression in colorectal tumors: evidence of different pathways in sporadic and ulcerative-colitis-associated carcinomas. *Am J Pathol* 1996; **149**: 1719-1726
- Itoh J**, de La Motte C, Strong SA, Levine AD, Fiocchi C. Decreased Bax expression by mucosal T cells favours resistance to apoptosis in Crohn's disease. *Gut* 2001; **49**: 35-41
- Cui YF**, Xia GW, Fu XB, Yang H, Peng RY, Zhang Y, Gu QY, Gao YB, Cui XM, Hu WH. Relationship between expression of Bax and Bcl-2 proteins and apoptosis in radiation compound wound healing of rats. *Chin J Traumatol* 2003; **6**: 135-138
- Nagata S**, Golstein P. The Fas death factor. *Science* 1995; **267**: 1449-1456
- Strater J**, Wellisch I, Riedl S, Walczak H, Koretz K, Tandara A, Krammer PH, Moller P. CD95 (APO-1/Fas)-mediated apoptosis in colon epithelial cells: a possible role in ulcerative colitis. *Gastroenterology* 1997; **113**: 160-167
- Iwamoto M**, Koji T, Makiyama K, Kobayashi N, Nakane PK. Apoptosis of crypt epithelial cells in ulcerative colitis. *J Pathol* 1996; **180**: 152-159
- Iwamoto M**, Makiyama K, Koji T, Kohno S, Nakane PK. Expression of Fas and Fas-ligand in epithelium of ulcerative colitis. *Nippon Rinsho* 1996; **54**: 1970-1974
- Mountz JD**, Zhou T, Su X, Wu J, Cheng J. The role of programmed cell death as an emerging new concept for the pathogenesis of autoimmune diseases. *Clin Immunol Immunopathol* 1996; **80**(3 Pt 2): S2-14
- Nagata S**. Fas and Fas ligand: a death factor and its receptor. *Adv Immunol* 1994; **57**: 129-144
- Yukawa M**, Iizuka M, Horie Y, Yoneyama K, Shirasaka T, Ito

- H, Komatsu M, Fukushima T, Watanabe S. Systemic and local evidence of increased Fas-mediated apoptosis in ulcerative colitis. *Int J Colorectal Dis* 2002; **17**: 70-76
- 43 **Coffey JC**, Bennett MW, Wang JH, O'Connell J, Neary P, Shanahan F, Redmond HP, Kirwan WO. Upregulation of Fas-Fas-L (CD95/CD95L)-mediated epithelial apoptosis—a putative role in pouchitis? *J Surg Res* 2001; **98**: 27-32
- 44 **Iwamoto M**, Makiyama K, Koji T, Kohno S, Nakane PK. Expression of Fas and Fas-ligand in epithelium of ulcerative colitis. *Nippon Rinsho* 1996; **54**: 1970-1974
- 45 **Moller P**, von Reyher U, Leithauser F, Strater J. CD95 (APO-1/Fas) and CD95-ligand (CD95L). Implications of these apoptosis mediating receptor/ligand systems in the pathogenesis of autoimmune diseases. *Verh Dtsch Ges Pathol* 1996; **80**: 12-22
- 46 **Wu HG**, Zhou LB, Shi DR, Liu SM, Liu HR, Zhang BM, Chen HP, Zhang LS. Morphological study on colonic pathology in ulcerative colitis treated by moxibustion. *World J Gastroenterol* 2000; **9**: 861-865
- 47 **Tsukada Y**, Nakamura T, Iimura M, Iizuka BE, Hayashi N. Cytokine profile in colonic mucosa of ulcerative colitis correlates with disease activity and response to granulocytapheresis. *Am J Gastroenterol* 2002; **97**: 2820-2828
- 48 **Banks C**, Bateman A, Payne R, Johnson P, Sheron N. Chemokine expression in IBD. Mucosal chemokine expression is unselectively increased in both ulcerative colitis and Crohn's disease. *J Pathol* 2003; **199**: 28-35
- 49 **Brown KA**, Back SJ, Ruchelli ED, Markowitz J, Mascarenhas M, Verma R, Piccoli DA, Baldassano RN. Lamina propria and circulating interleukin-6 in newly diagnosed pediatric inflammatory bowel disease patients. *Am J Gastroenterol* 2002; **97**: 2603-2608
- 50 **Ueyamam H**, Kiyohara T, Sawada N, Isozaki K, Kitamura S, Kondo S, Miyagawa J, Kanayama S, Shinomura Y, Ishikawa H, Ohtani T, Nezu R, Nagata S, Matsuzawa Y. Hish Fas ligand expression on lymphocytes in lesions of ulcerative colitis. *Gut* 1998; **43**: 48-55
- 51 **Strater J**, Wellisch I, Riedl S, Walczak H, Koretz K, Tandara A, Krammer PH, Moller P. CD95(APO-1/Fas)-mediated apoptosis in colon epithelial cells: a possible role in ulcerative colitis. *Gastroenterology* 1997; **113**: 160-167
- 52 **Suzuki A**, Sugimura K, Ohtsuka K, Hasegawa K, Suzuki K, Ishizuka K, Mochizuki T, Honma T, Narisawa R, Asakura H. Fas/Fas ligand expression and characteristics of primed CD45RO+ T cells in the inflamed mucosa of ulcerative colitis. *Scand J Gastroenterol* 2000; **35**: 1278-1283
- 53 **Wu HG**, Chen HP, Shi Z, Hua XG, Zhao C. Clinical study of the treatment of chronic ulcerative colitis with moxibustion. *Int J Clin Acupunct* 1999; **1**: 26-28
- 54 **Wu HG**, Shi Z, Zhou LB, Pan YY, Tan WL. Acupuncture and moxibustion's effect on cytokine of ulcerative colitis rat. *Int J Clin Acupunct* 2000; **3**: 43-48
- 55 **Wu HG**, Zhou LB, Huang C, Pan YY, Chen HP, Shi Z, Hua XG. Gene expression of cytokines in acupuncture and moxibustion treatment for ulcerative colitis in rats. *Huaren Xiaohua Zazhi* 1998; **6**: 853-855
- 56 **Wu HG**, Chen HP, Zhou LB, Pan YY, Huang C, Shi Z. molecule mechanism of acupuncture and moxibustion's therapy effect on ulcerative colitis rat. *Shanghai Zhenjiu Zazhi* 1998; **17**: 30

Edited by Wang XL



# Effects of tumor necrosis factor, endothelin and nitric oxide on hyperdynamic circulation of rats with acute and chronic portal hypertension

Ji-Jian Wang, Gen-Wu Gao, Ren-Zhong Gao, Chang-An Liu, Xiong Ding, Zhen-Xiang Yao

**Ji-Jian Wang, Gen-Wu Gao, Chang-An Liu, Xiong Ding**, Department of General Surgery, Second Affiliated Hospital of Chongqing University of Medical Sciences, Chongqing 400010, China

**Ren-Zhong Gao**, Department of Emergency, Maroondah Hospital, Melbourne, Australia

**Zhen-Xiang Yao**, Department of General Surgery, First Affiliated Hospital of Chongqing University of Medical Sciences, Chongqing 400016, China

**Supported by** Science Foundation of Chongqing Health Bureau, No. 97-09

**Correspondence to:** Ji-Jian Wang, Department of General Surgery, Second Affiliated Hospital of Chongqing University of Medical Sciences, 74 Linjing Road, Chongqing 400010, China. wangjijian11@hotmail.com

**Telephone:** +86-23-67764551 **Fax:** +86-23-67652167

**Received:** 2002-10-30 **Accepted:** 2002-11-25

## Abstract

**AIM:** To evaluate the effect of tumor necrosis factor (TNF), endothelin (ET) and nitric oxide (NO) on hyperdynamic circulation (HC) of rats with acute and chronic portal hypertension (PHT).

**METHODS:** Chronic portal hypertension was induced in Wistar rats by injection of carbon tetrachloride. After two weeks of cirrhosis formation, L-NMMA (25 mg/kg) was injected into one group of cirrhotic rats via femoral vein and the experiment was begun immediately. Another group of cirrhotic rats was injected with anti-rat TNF $\alpha$  (300 mg/kg) via abdominal cavity twice within 48 h and the experiment was performed 24 h after the second injection. The blood concentrations of TNF $\alpha$ , ET-1 and NO in portal vein and the nitric oxide synthase (NOS) activity in hepatic tissue were determined pre-and post-injection of anti-rat TNF $\alpha$  or L-NMMA. Stroke volume (SV), cardiac output (CO), portal pressure (PP), superior mesenteric artery blood flow (SMA flow) and iliac artery blood flow (IAflow) were measured simultaneously. Acute portal hypertension was established in Wistar rats by partial portal-vein ligation (PVL). The parameters mentioned above were determined at 0.5 h, 24 h, 48 h, 72 h and 120 h after PVL. After the formation of stable PHT, the PVL rats were injected with anti-rat TNF $\alpha$  or L-NMMA according to different groups, the parameters mentioned above were also determined.

**RESULTS:** In cirrhotic rats, the blood levels of TNF $\alpha$ , NO in portal vein and the liver NOS activity were significantly increased ( $P < 0.05$ ) while the blood level of ET-1 was not statistically different ( $P > 0.05$ ) from the control animals (477.67 $\pm$ 83.81 pg/mL vs 48.87 $\pm$ 32.79 pg/mL, 278.41 $\pm$ 20.11  $\mu$ mol/L vs 113.28 $\pm$ 14.51  $\mu$ mol/L, 1.81 $\pm$ 0.06 u/mg $\cdot$ prot vs 0.87 $\pm$ 0.03 u/mg $\cdot$ prot and 14.33 $\pm$ 4.42 pg/mL vs 8.72 $\pm$ 0.79 pg/mL, respectively). After injection of anti-rat TNF $\alpha$ , the blood level of TNF $\alpha$  was lower than that in controls (15.17 $\pm$ 18.79 pg/mL vs 48.87 $\pm$ 32.79 pg/mL). The blood level

of NO and the liver NOS activity were significantly decreased, but still higher than those of the controls. The blood level of ET-1 was not significantly changed. PP, SV, CO, SMAflow and IAflow were ameliorated. After injection of L-NMMA, the blood level of NO and the liver NOS activity were recovered to those of the controls. PP and CO were also recovered to those of the controls. SV, SMAflow and IAflow were ameliorated. In PVL rats, the blood levels of TNF $\alpha$ , NO in portal vein and the liver NOS activity were gradually increased and reached the highest levels at 48 h after PVL. The blood level of ET-1 among different staged animals was not significantly different from the control animals. PP among different staged animals (2.4 $\pm$ 0.18 kPa at 0.5 h, 1.56 $\pm$ 0.08 kPa at 24 h, 1.74 $\pm$ 0.1 kPa at 48 h, 2.38 $\pm$ 0.05 kPa at 72 h, 2.39 $\pm$ 0.16 kPa at 120 h) was significantly higher than that in controls (0.9 $\pm$ 0.16 kPa). After injection of anti-rat TNF $\alpha$  in 72 h PVL rats, the blood level of TNF $\alpha$  was lower than that in controls (14 $\pm$ 14 pg/mL vs 48.87 $\pm$ 32.79 pg/mL). The blood level of NO and the liver NOS activity were significantly decreased, but still higher than those of the controls. The blood level of ET-1 was not significantly changed. PP was decreased from 2.38 $\pm$ 0.05 kPa to 1.68 $\pm$ 0.12 kPa, but significantly higher than that in controls. SV, CO, SMAflow and IAflow were ameliorated. After injection of L-NMMA in 72 h PVL rats, the blood level of NO and the liver NOS activity were recovered to those of the controls. PP, SV, CO, SMAflow and IAflow were also recovered to those of the controls.

**CONCLUSION:** NO plays a critical role in the development and maintenance of HC in acute PHT and is a key factor for maintenance of HC in chronic PHT. TNF $\alpha$  may not participate in the hemodynamic changes of HC directly, while play an indirect role by inducing the production of NO through activating NOS. No evidence that circulating ET-1 plays a role in both models of portal hypertension has been found.

Wang JJ, Gao GW, Gao RZ, Liu CA, Ding X, Yao ZX. Effects of tumor necrosis factor, endothelin and nitric oxide on hyperdynamic circulation of rats with acute and chronic portal hypertension. *World J Gastroenterol* 2004; 10(5): 689-693  
<http://www.wjgnet.com/1007-9327/10/689.asp>

## INTRODUCTION

Associated with hyperdynamic circulatory syndrome (HCS), the portal hypertension (PHT) is characterized by systemic vasodilatation, increase of plasma volume, cardiac output and regional blood flow<sup>[1-8]</sup>. Although it is most likely initiated by vasodilatation resulted from an increase of vasodilator activity<sup>[9]</sup>, the etiology of HCS is still controversial. Two potent vasodilators, endogenous nitric oxide (NO) and tumor necrosis factor (TNF) may play important roles in the pathogenesis of hemodynamic changes of PHT<sup>[1,10]</sup>. As a powerful vasoconstrictor, endothelin (ET) could influence the

pathogenesis of hemodynamic changes of PHT as well<sup>[5,11-15]</sup>. Since ET has contradictory effect on blood vessels in comparison with the former two, it is hard to imagine that they synergistically take part in the hemodynamic changes. It is thus necessary to find out what kind of role the three factors play in the pathogenesis of HCS, respectively.

## MATERIALS AND METHODS

### Reagents

Carbon tetrachloride was purchased from Chongqing Chemical Reagents Factory (Chongqing, China). A rabbit anti-rat TNF $\alpha$  antibody was purchased from PharMingen Company (USA). N<sup>G</sup>-methyl-L-arginine (L-NMMA) and endothelin EIA kit were purchased from Cayman Company (USA). Rat TNF $\alpha$  ELISA kit was purchased from Endogen Company (USA). NO and NOS determining kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

### Animal model of acute PHT (aPHT)

Partial portal vein ligation (PVL) was performed to establish the aPHT model as described previously<sup>[10]</sup>. In brief, male Wistar rats (220-280 g, offered by the Animal Center of Chongqing University of Medical Sciences) had free access to water and standard rat chow. After fasted overnight, the rats were anesthetized with pentobarbital intra- abdominally at a dose of 60 mg/kg. The portal vein was isolated and two ligatures were placed around both the portal vein and a 16-gauge blunt-end needle. One ligature was placed 1 mm distal to the bifurcation of portal vein and the other ligature was placed 1-2 mm to the input point of splenic vein. The needle was ligated together with the portal vein and immediately removed to allow the portal vein to expand to the limit imposed by the ligature. The abdomen was closed. In sham-operated rats, surgery consisted of dissection and visual inspection of the portal vein without ligation.

### Animal model of chronic PHT (cPHT)

Carbon tetrachloride induced cirrhosis was made as the cPHT model. Male Wistar rats (150-200 g, provided by the Animal Center of Chongqing University of Medical Sciences) had free access to standard rat chow and 100mL/L alcohol. Cirrhosis model was established by injection with 600mL/L carbon tetrachloride mixed with paraffin liquid subcutaneously at a dose of 0.3 mL/100g at the lateral abdomen of both sides, twice a week for 17 times. The rats were allowed to stabilize for 2 weeks.

### Determination of hemodynamic indexes

**Stroke volume (SV) and cardiac output (CO)** Ultrasonic probe of HEWLETT PACKARD 5500 type ultrasonic instrument (USA) was placed on the parasternum of rats at the left ventricle long axis and mitral valve level and then exchanged by M type ultrasonic image. The inner computer system of this instrument would calculate and display the data we needed.

**Superior mesenteric artery (SMA) and iliac artery (IA) blood flow** SMA and IA were isolated and embraced by a cuff of electromagnetic flowmeter (NIHON KHDEN, Japan) respectively. Its blood flow was determined while blood passed a photoelectric sensor.

**Portal pressure (PP) and right atrial pressure (RAP)** A 7 gauge needle was penetrated into portal vein in the direction of liver and a catheter was inserted through the internal jugular vein into the right atrium and connected to a pressure transducer respectively. PP and RAP were recorded with a four-channel physiometer (NIHON KOHDEN, Japan).

**Mean arterial pressure (MAP)** Rat's tail was placed in a photoelectric channel and MAP was determined with an RBP-1 type blood pressometer.

**Calculation of superior mesenteric artery vascular resistance (VR<sub>SMA</sub>) and iliac artery vascular resistance (VR<sub>IA</sub>)** VR<sub>SMA</sub> and VR<sub>IA</sub> were calculated according to the following formula reported by Colombato *et al*<sup>[16]</sup>.

$$VR_{SMA}(\text{kPa}/\text{L} \cdot \text{min}) = \frac{MAP-PP}{SMA-flow}$$

$$VR_{IA}(\text{kPa} \cdot / \text{L} \cdot \text{min}) = \frac{MAP-RAP}{IA-flow}$$

### Serum levels of TNF $\alpha$ , ET-1 and NO and hepatic activity of NOS

Blood was obtained from the portal vein at the time of sacrifice. Hepatic tissue was obtained from the left lobe of rat's liver. Serum level of TNF $\alpha$  was measured by ELISA according to the manufacturer's instructions (PharMingen Co., USA). Serum samples were analyzed for ET-1 content by EIA according to the manufacturer's instructions (Cayman Co., USA). Serum samples and hepatic tissues were analyzed for NO content and NOS activity according to the manufacturer's instructions by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

### Experimental protocol

The rats involved in this experiment were divided into experimental group, treated group and control group. Fifty aPHT(PVL) rats were divided into five staged subgroups (0.5 h, 24 h, 48 h, 72 h and 120 h after PVL, 10 rats each group) and 10 cPHT rats were used as the experimental group. Twenty cPHT rats and 20 aPHT rats were divided into two groups (10 rats each group) respectively as the treatment group, and the method of treatment was as following. Ten cPHT rats were injected with anti-rat TNF $\alpha$  twice within 48 h and the experiment was performed 24 h after the second injection and 10 aPHT rats were injected with anti-rat TNF $\alpha$  at 0.5 h and 48 h after PVL respectively and the experiment was performed at 72 h after PVL (one dose of 300  $\mu$ g/kg, via intra-abdominal cavity). The other 10 aPHT rats and 10 cPHT rats were injected with L-NMMA (25 mg/kg) through femoral vein 72 h after PVL and the experiment was performed immediately. Ten normal rats and 10 sham operated rats were used as the cPHT and the aPHT control groups respectively. The following parameters were determined, namely the hemodynamic indexes, serum levels of TNF $\alpha$ , ET-1 and NO, hepatic NOS activity.

### Statistical analysis

Data were expressed as mean $\pm$ SD and analysis of variance was performed using SPSS 8.0 software. Differences between groups were analyzed using *t*-test. One-way analysis of variance was used for multiple comparisons, and Newman-Keuls test was used for intra-group comparisons. *P*<0.05 was considered statistically significant.

## RESULTS

### Hemodynamic change

In PVL rats, SV, CO, PP, SMAflow and IAflow were significantly increased from 0.16 $\pm$ 0.04 mL/s, 0.058 $\pm$ 0.008 L/min, 0.9 $\pm$ 0.16 kPa, 8.24 $\pm$ 1.16 mL $\cdot$ min<sup>-1</sup> and 10 $\pm$ 0.89 mL $\cdot$ min<sup>-1</sup> to 0.27 $\pm$ 0.02 mL/s, 0.113 $\pm$ 0.004 L/min, 1.74 $\pm$ 0.1 kPa, 17.58 $\pm$ 0.7 mL/min and 20.42 $\pm$ 1.07 mL/min respectively at 48 h after PVL (*P*<0.05). VR<sub>SMA</sub> and VR<sub>IA</sub> were significantly decreased from 15.57 $\pm$ 2.75 kPaL/min and 13.58 $\pm$ 2.19 kPaL/min to 5.96 $\pm$ 0.35 kPaL/min and 5.62 $\pm$ 0.33 kPaL/min respectively at 48 h after PVL (*P*<0.05).

These hemodynamic variables no matter increased or decreased, all reached a maximal level at 72 h after PVL ( $P<0.05$ ) and did not change thereafter ( $P>0.05$ ). In cirrhotic rats, SV, CO, PP, SMAflow and IAflow were significantly increased from  $0.162\pm0.04$  mL/s,  $0.058\pm0.017$  L/min,  $0.91\pm0.16$  kPa,  $8.42\pm1.16$  mL/min and  $10\pm0.89$  mL/min to  $0.59\pm0.06$  mL/s,  $0.159\pm0.031$  L/min,  $2.26\pm0.39$  kPa,  $27\pm3.19$  mL/min and  $27.33\pm1.21$  mL/min respectively at the time of cirrhosis formation ( $P<0.05$ ).  $VR_{SMA}$  and  $VR_{IA}$  were significantly decreased from  $15.57\pm2.74$  kPaL/min and  $13.58\pm2.19$  kPaL/min to  $4.07\pm0.43$  kPaL/min and  $4.44\pm0.13$  kPaL/min respectively at the time of cirrhosis formation ( $P<0.05$ ).

#### Alteration of serum TNF $\alpha$ , ET-1 and NO and hepatic NOS activity

Serum TNF $\alpha$  and NO levels and NOS activity were significantly increased at 24 h compared with those of control ( $P<0.05$ ) and reached a maximal level at 48 h after PVL and did not change thereafter. An increase of serum ET-1 levels was also observed at different time points post PVL, however, all of them did not reach the significant level as compared with control ( $P>0.05$ ). The serum TNF $\alpha$  and NO levels were significantly increased compared with control in cirrhotic rats ( $P<0.05$ ) and their increment was markedly greater than those rats 48 h after PVL ( $P<0.05$ ). The NOS activity was also markedly increased ( $P<0.05$ ) and the serum ET-1 level was slightly increased, but did not reach the significant level ( $P>0.05$ ) compared with control in cirrhotic rats (Table 1).

#### Effects of anti-rat TNF $\alpha$ and L-NMMA on serum TNF $\alpha$ , ET-1 and NO and hepatic NOS

The serum level of TNF $\alpha$  was markedly decreased and lower than that of controls in both rat models, and the serum level of NO and hepatic NOS activity were significantly decreased,

but still markedly greater than those of controls. Injection of anti-rat TNF $\alpha$  had no effect on the level of ET-1. The serum level of NO and hepatic NOS activity were significantly decreased to the levels of the controls in both rat models after injection of L-NMMA (Table 2).

**Table 1** Serum levels of TNF $\alpha$ , ET-1 and NO and hepatic NOS activities in PVL and cPHT rats

Groups	TNF $\alpha$ (pg/min)	ET-1 (pg/min)	NO ( $\mu$ mol/L)	NOS (u/mg·prot)
Control	48.67 $\pm$ 32.79	8.72 $\pm$ 0.79	113 $\pm$ 15	0.9 $\pm$ 0.03
aPHT				
PVL0.5 h	50 $\pm$ 15	13.4 $\pm$ 2.6	128.64 $\pm$ 18.29	0.86 $\pm$ 0.14
PVL24 h	328 $\pm$ 100 <sup>ac</sup>	12.8 $\pm$ 5.7	169.40 $\pm$ 21.07 <sup>ac</sup>	1.45 $\pm$ 0.17 <sup>ac</sup>
PVL48 h	428 $\pm$ 69 <sup>a</sup>	13.5 $\pm$ 2.6	223.71 $\pm$ 35.44 <sup>a</sup>	1.7 $\pm$ 0.12 <sup>a</sup>
PVL72 h	416 $\pm$ 48 <sup>a</sup>	13.1 $\pm$ 3.2	215.49 $\pm$ 12.75 <sup>a</sup>	1.67 $\pm$ 0.16 <sup>a</sup>
PVL120 h	425 $\pm$ 49 <sup>a</sup>	13.4 $\pm$ 3.5	225.36 $\pm$ 18.66 <sup>a</sup>	1.7 $\pm$ 0.12 <sup>a</sup>
cPHT	477.67 $\pm$ 83.81 <sup>ae</sup>	14.33 $\pm$ 4.42	278.41 $\pm$ 20.11 <sup>ae</sup>	1.81 $\pm$ 0.06 <sup>a</sup>

<sup>a</sup> $P<0.05$  vs control, <sup>c</sup> $P<0.05$  PVL 24 h group compared with PVL 48 h, 72 h, 120 h and cPHT groups respectively, <sup>e</sup> $P<0.05$  cPHT group compared with PVL 48 h, 72 h, 120 h groups respectively.

#### Effects of anti-rat TNF $\alpha$ and L-NMMA on hemodynamic variables

In both rat models, SV, CO, PP, SMAflow and IAflow were significantly decreased, but were still markedly higher than those of the controls. However,  $VR_{SMA}$  and  $VR_{IA}$  were significantly increased but still markedly lower than those of the controls after injection of anti-rat TNF $\alpha$ . In PVL rats, SV, CO, PP, SMAflow, IAflow,  $VR_{SMA}$  and  $VR_{IA}$  were all recovered to the levels of the controls after injection of L-NMMA. In cPHT rats, CO and PP were exclusively recovered to the levels of controls. SV, SMAflow, IAflow,  $VR_{SMA}$  and  $VR_{IA}$  were markedly increased or decreased, but still significantly different from those of the controls after injection of L-NMMA (Table 3).

**Table 2** Effects of anti-rat TNF $\alpha$  and L-NMMA on levels of TNF $\alpha$ , ET-1 and hepatic NO and NOS activity in PVL and cPHT rats

Groups	TNF $\alpha$ (pg/mL)	ET-1 (pg/mL)	NO ( $\mu$ mol/L)	NOS (u/mg·prot)
Control	48.67 $\pm$ 32.29	8.72 $\pm$ 0.79	113 $\pm$ 15	0.9 $\pm$ 0.03
cPHT	477.67 $\pm$ 83.8 <sup>ac</sup>	14.33 $\pm$ 4.42	278.41 $\pm$ 20.11 <sup>ac</sup>	1.81 $\pm$ 0.06 <sup>ac</sup>
cPHT+anti-rat TNF $\alpha$	15.17 $\pm$ 18.79 <sup>a</sup>	14.33 $\pm$ 4.42	190.61 $\pm$ 10.9 <sup>a</sup>	1.39 $\pm$ 0.04 <sup>a</sup>
cPHT+L-NMMA	—	—	119.18 $\pm$ 11.51 <sup>e</sup>	4.92 $\pm$ 0.03 <sup>e</sup>
PVL72 h	416 $\pm$ 48 <sup>ag</sup>	13.1 $\pm$ 3.2	215.49 $\pm$ 12.75 <sup>ag</sup>	1.67 $\pm$ 0.16 <sup>ag</sup>
PVL72 h+anti-rat TNF $\alpha$	14 $\pm$ 14 <sup>a</sup>	13.5 $\pm$ 2.6	178.59 $\pm$ 14.61 <sup>a</sup>	1.34 $\pm$ 0.09 <sup>a</sup>
PVL72 h+L-NMMA	—	—	104.61 $\pm$ 18 <sup>i</sup>	0.95 $\pm$ 0.08 <sup>i</sup>

<sup>a</sup> $P<0.05$  vs control, <sup>c</sup> $P<0.05$  cPHT group compared with cPHT+anti-rat TNF $\alpha$  and cPHT+L-NMMA groups respectively, <sup>e</sup> $P<0.05$  cPHT+anti-rat TNF $\alpha$  compared with cPHT+L-NMMA groups, <sup>g</sup> $P<0.05$  PVL72 h group compared with PVL72 h+anti-rat TNF $\alpha$  and PVL72 h+L-NMMA groups respectively, <sup>i</sup> $P<0.05$  PVL72 h+anti-rat TNF $\alpha$  compared with PVL72 h+L-NMMA.

**Table 3** Effects of anti-rat TNF $\alpha$  and L-NMMA on hemodynamic variables

Groups	SV (mL/s)	CO (L/min)	PP (kPa)	SMAflow (mL/min)	IAflow (mL/min)	$VR_{SMA}$ (kPa/.L·min)	$VR_{IA}$ (kPa/.L·min)
Control	0.162 $\pm$ 0.04	0.05 $\pm$ 0.017	0.91 $\pm$ 0.16	8.42 $\pm$ 1.16	10 $\pm$ 0.89	15.57 $\pm$ 2.74	13.58 $\pm$ 2.19
cPHT	0.59 $\pm$ 0.06 <sup>ac</sup>	0.159 $\pm$ 0.031 <sup>ac</sup>	2.26 $\pm$ 0.34 <sup>ac</sup>	27 $\pm$ 3.19 <sup>ac</sup>	27.33 $\pm$ 1.21 <sup>ac</sup>	4.07 $\pm$ 0.43 <sup>ac</sup>	4.44 $\pm$ 0.51 <sup>ac</sup>
CPHT+anti-rat TNF $\alpha$	0.39 $\pm$ 0.08 <sup>a</sup>	0.138 $\pm$ 0.029 <sup>a</sup>	1.53 $\pm$ 0.13 <sup>a</sup>	20.75 $\pm$ 1.92 <sup>a</sup>	24.15 $\pm$ 1.67 <sup>a</sup>	5.56 $\pm$ 0.59 <sup>a</sup>	5.21 $\pm$ 0.51 <sup>a</sup>
cPHT+L-NMMA	0.32 $\pm$ 0.02 <sup>a</sup>	0.076 $\pm$ 0.005 <sup>e</sup>	1.12 $\pm$ 0.08 <sup>e</sup>	12.75 $\pm$ 0.82 <sup>ac</sup>	16.08 $\pm$ 0.74 <sup>ae</sup>	10.13 $\pm$ 0.26 <sup>ac</sup>	8.84 $\pm$ 0.66 <sup>ae</sup>
PVL72 h	0.42 $\pm$ 0.03 <sup>ag</sup>	0.143 $\pm$ 0.029 <sup>ag</sup>	2.38 $\pm$ 0.05 <sup>ag</sup>	23.27 $\pm$ 1.52 <sup>ag</sup>	25.43 $\pm$ 1.44 <sup>ag</sup>	4.44 $\pm$ 0.28 <sup>ag</sup>	4.54 $\pm$ 0.32 <sup>ag</sup>
PVL72 h+anti-rat TNF $\alpha$	0.33 $\pm$ 0.02 <sup>a</sup>	0.127 $\pm$ 0.008 <sup>a</sup>	1.68 $\pm$ 0.12 <sup>a</sup>	19.3 $\pm$ 1.1 <sup>a</sup>	20.7 $\pm$ 2 <sup>a</sup>	5.9 $\pm$ 0.1 <sup>a</sup>	6.0 $\pm$ 0.5 <sup>a</sup>
PVL72 h+L-NMMA	0.24 $\pm$ 0.04 <sup>i</sup>	0.072 $\pm$ 0.013 <sup>i</sup>	0.85 $\pm$ 0.15 <sup>i</sup>	9.82 $\pm$ 0.96 <sup>i</sup>	10.77 $\pm$ 1.11 <sup>i</sup>	12.58 $\pm$ 0.93 <sup>i</sup>	12.42 $\pm$ 0.99 <sup>i</sup>

<sup>a</sup> $P<0.05$  vs control, <sup>c</sup> $P<0.05$  cPHT group compared with cPHT+anti-rat TNF $\alpha$  and cPHT+L-NMMA groups respectively, <sup>e</sup> $P<0.05$  cPHT+anti-rat TNF $\alpha$  compared with cPHT+L-NMMA groups, <sup>g</sup> $P<0.05$  PVL 72 h group compared with PVL 72 h+anti-rat TNF $\alpha$  and PVL 72 h+L-NMMA groups respectively, <sup>i</sup> $P<0.05$  PVL 72 h+anti-rat TNF $\alpha$  compared with PVL 72 h+L-NMMA.

## DISCUSSION

In our study, HCS was observed in rats with acute and chronic PHT, and characterized by the increase of SV, CO, regional blood flow and PP, as well as the decrease of peripheral and splanchnic vascular resistance. This agreed with a lot of literature<sup>[1-8]</sup>. Lopez-Talavera *et al*<sup>[9,10]</sup> studied the correlation between hemodynamic changes and TNF $\alpha$  on days 5, 13 and 14 after PVL, and found that TNF $\alpha$  might play a role in HCS of portal hypertension. In this study, we found that the serum level of TNF $\alpha$  in portal vein was markedly increased at 24 h, reached a peak at 48 h and maintained stable thereafter in PVL rats. Whereas, the obvious hemodynamic changes occurred at 48 h and HCS was induced at 72 h, about 24 h later than the obvious increase in TNF $\alpha$  level. The serum level of TNF $\alpha$  was much more higher in cPHT rats than that in rats 48 h after PVL. There was no obvious difference between the hemodynamic indexes of both groups. Therefore, we speculated that TNF $\alpha$  might play a role in the early stage of HCS, and that overproduction of TNF $\alpha$  might have a mild effect on hemodynamic changes. In the anti-rat TNF $\alpha$  experiment, we found that the serum level of TNF $\alpha$  was lower than that of the controls and the effect of TNF $\alpha$  was completely inhibited by the injected anti-rat TNF $\alpha$ . Although the hemodynamics was significantly changed, it still had a remarkable difference in comparison with the controls. In other words, HCS was improved and a new HCS balanced on a lower basis formed. At the same time, the NO levels and hepatic NOS activity in rats with hepatic cirrhosis and PVL were decreased by 20-25% and 15-30%, respectively. Kaviani *et al*<sup>[17]</sup> revealed that after gastric strips from PVL rats were incubated with TNF $\alpha$  neutralizing antibody, inducible NOS mRNA expression was significantly decreased by 40%, 70%, and 80% after 1, 2, and 6 h. This suggested that the vasoactive effect of TNF $\alpha$  itself on the development and formation of HCS in portal hypertension was little, and that corresponding hemodynamic changes after injection of TNF $\alpha$  antibody were due to the elimination of TNF $\alpha$  activation on NOS and the decreased production of NO. This conclusion disagreed with the report of Lopez-Talavera *et al*<sup>[9]</sup> that anti-rat TNF $\alpha$  treatment of rats after PVL significantly inhibited hyperdynamic circulation and reduced portal pressure. It was also inconsistent with the report of Munoz *et al*<sup>[18]</sup>. In their experiment, anti-TNF $\alpha$  polyclonal antibodies were injected into rats before and 4 days after portal vein stenosis (PVS) (short-term inhibition) and at 24 h and 4, 7, 10 d after PVS (long-term inhibition). After a short-term inhibition or a long-term inhibition, portal pressure kept unchanged. Tabrizchi<sup>[19]</sup> found that cardiac output, blood pressure and mean circulatory filling pressure were significantly reduced, but the arterial resistance increased following treatment with TNF $\alpha$  in anaesthetized rats. This, obviously, did not accord with the features of HCS at PHT, and also suggested that TNF $\alpha$  did not directly take part in the hemodynamic changes at PHT.

In our study, we found the serum NO level and hepatic NOS activity in the two animal models with portal hypertension were decreased by 20-25% and 15-30% respectively after injection of anti-rat TNF $\alpha$  antibody. This suggested that the increase of serum NO level was stimulated by the combination of TNF $\alpha$  and other media such as IL-6 and INF $\alpha$ , etc., which agreed with what was reported<sup>[10,20,21]</sup>. Wiest *et al*<sup>[21]</sup> reported that upregulation of eNOS release and increase of NO by SMA endothelium occurred before the development of hyperdynamic splanchnic circulation, suggesting a primary role of NO in the pathogenesis of arterial vasodilatation. But the results reported by Albornoz *et al*<sup>[22]</sup> disagreed with those of ours. Their results showed that dexamethasone (an inhibitor of the expression of the iNOS) administration did not modify

systemic and splanchnic hemodynamic parameters in endotoxemic cirrhotic rats and suggested that stimulation of iNOS might not play a role in increasing NO production in portal hypertension. We found that the NO level in portal vein and the liver NOS activity were significantly decreased to the level of controls by injecting L-NMMA in cirrhotic rats and in rats 72 h after PVL. In PVL rats, the hemodynamics was recovered to the controls. In cirrhotic rats, the PP was also recovered to the control. But the SV was still significantly greater than that of control and the systemic vasodilatation was not recovered to the state of control. These results suggested that NO played a critical role in the development and maintenance of HCS in acute PHT and was a key factor in maintenance of HCS in chronic PHT. This conclusion was consistent with those of most authors<sup>[1,5-7,23-26]</sup>. In patients with chronic portal vein hypertension, since the tissue structure of vascular wall was changed due to the long term dilatation of systemic blood vessels, the dilated blood vessels would be hard to recover, even if the effect of vasodilators had been completely eliminated.

Elevated ET-1 level in blood and its active role in portal hypertension in cirrhotic patients and a variety of animal models have been reported by many authors<sup>[13-16,27-29]</sup>. Nevertheless, Poo *et al*<sup>[30]</sup> reported that the liver paracrine ET system did not play a major role in the pathogenesis of portal hypertension, but took part in the development of liver fibrogenesis. Varagic *et al*<sup>[31]</sup> reported that circulating endothelin-1 did not play a role in spontaneously hypertensive rats. In this study, the blood level of ET-1 in portal vein was mildly increased but not significantly higher in comparison with the controls in cirrhotic and PVL rats. This finding suggested that ET-1 might not play a role in the development of hemodynamic abnormalities in PHT. It might keep the tension of blood vessels and antagonize the effect of vasodilators. Therefore, ET-1 may have a regulating effect on the vasodilatation and vascular refilling. Our finding was consistent with Poo *et al*<sup>[30]</sup>, but inconsistent with the other authors<sup>[12-15,27-29]</sup>.

Based on the result of a combination study of TNF $\alpha$ , NO and ET, we draw a conclusion that TNF $\alpha$  may not directly participate in the hemodynamic changes of HCS, while exert an indirect effect by inducing the production of NO. NO is the primary factor for forming and maintaining HCS at PHT. ET does not directly take part in the hemodynamic changes of PHT either, while keeps the tension of blood vessel and prevents it from overdilating under the effect of vasodilatation factors.

## REFERENCES

- Huang YQ, Xiao SD, Zhang DZ, Mo JZ. Nitric oxide synthase distribution in esophageal mucosa and hemodynamic changes in rats with cirrhosis. *World J Gastroenterol* 1999; **5**: 213-216
- Trevisani F, Sica G, Mainqua P, Santese G, Notariis SD, Caraceni P, Domenicali M, Zaca F, Grazi GL, Mazziotti A, Cavallari A, Bernardi M. Autonomic dysfunction and hyperdynamic circulation in cirrhosis with ascites. *Hepatology* 1999; **30**: 1387-1392
- Zhu JY, Leng XS, Wang D, Du RY. Effects of somatostatin on splanchnic hemodynamics in cirrhotic patients with portal hypertension. *World J Gastroenterol* 2000; **6**: 143-144
- Lebrec D, Moreau R. Pathogenesis of portal hypertension. *Eur J Gastroenterol Hepatol* 2001; **13**: 309-311
- Tsugawa K, Hashizume M, Migou S, Kishihara F, Kawnaka H, Tomikawa M, Tanoue K, Sugimachi K. Role of nitric oxide and endothelin-1 in a portal hypertensive rat model. *Scand J Gastroenterol* 2000; **35**: 1097-1105
- Howe LM, Boothe DM, Slater MR, Boothe HW, Wilkie S. Nitric oxide generation in a rat model of acute portal hypertension. *Am J Vet Res* 2000; **61**: 1173-1177
- Bi XJ, Chen MH, Wang JH, Chen J. Effect of endotoxin on portal hemodynamic in rats. *World J Gastroenterol* 2002; **8**: 528-530

- 8 **Møller S**, Bendtsen F, Henriksen JH. Splanchnic and systemic hemodynamic derangement in decompensated cirrhosis. *Can J Gastroenterol* 2001; **15**: 94-106
- 9 **Lopez-Talavera JC**, Merrill WW, Groszmann RJ. Tumor necrosis factor  $\alpha$ : a major contributor to the hyperdynamic circulation in prehepatic portal-hypertensive rats. *Gastroenterology* 1995; **108**: 761-767
- 10 **Lopez-Talavera JC**, Cadelina G, Olchowski J, Merrill W, Groszmann RJ. Thalidomide inhibits tumor necrosis factor  $\alpha$ , decreases nitric oxide synthesis, and ameliorates the hyperdynamic circulatory syndrome in portal-hypertensive rats. *Hepatology* 1996; **23**: 1616-1621
- 11 **Liu F**, Li JX, Li CM, Leng XS. Plasma endothelin in patients with endotoxemia and dynamic comparison between vasoconstrictor and vasodilator in cirrhotic patients. *World J Gastroenterol* 2001; **7**: 126-127
- 12 **Chan CC**, Wang SS, Lee FY, Chang FY, Lin HC, Chu CJ, Chen CT, Huang HC, Lee SD. Endothelin-1 induces vasoconstriction on portal-systemic collaterals of portal hypertensive rats. *Hepatology* 2001; **33**: 816-820
- 13 **Nagasue N**, Dhra DK, Yamanoi A, Emi Y, Udagawa J, Yamamoto A, Tachibana M, Kubota H, Kohno H, Harada T. Production and release of endothelin-1 from the gut and spleen in portal hypertension due to cirrhosis. *Hepatology* 2000; **31**: 1107-1114
- 14 **Gottardi AD**, Shaw S, Sagesser H, Reichen J. Type A, but not type B, endothelin receptor antagonists significantly decrease portal pressure in portal hypertensive rats. *J Hepatol* 2000; **33**: 733-737
- 15 **Yokoyama Y**, Wawrzyniak A, Baveja R, Sonin N, Clemens MG, Zhang JX. Altered endothelin receptor expression in prehepatic portal hypertension predisposes the liver to microcirculatory dysfunction in rats. *J Hepatol* 2001; **35**: 29-36
- 16 **Colombato LA**, Albillos A, Groszmann RJ. Temporal relationship of peripheral vasodilatation, plasma volume expansion and the hyperdynamic circulatory state in portal-hypertensive rats. *Hepatology* 1991; **15**: 323-328
- 17 **Kaviani A**, Ohta M, Itani R, Sander F, Tarnawski AS, Sarfeh II. Tumor necrosis factor- $\alpha$  regulates inducible nitric oxide synthase gene expression in the portal hypertensive gastric mucosa of the rat. *J Gastrointest Surg* 1997; **1**: 371-376
- 18 **Munoz J**, Albillos A, Perez-Paramo M, Rossi I, Alvarez-Mon M. Factors mediating the hemodynamic effects of tumor necrosis factor- $\alpha$  in portal hypertensive rats. *Am J Physiol* 1999; **276**(3 Pt 1): G687-693
- 19 **Tabrizchi R**. The influence of tumour necrosis factor- $\alpha$  on the cardiovascular system of anaesthetized rats. *Naunyn Schmiedebergs Arch Pharmacol* 2001; **363**: 307-321
- 20 **Zhang GL**, Wang YH, Teng HL, Lin ZB. Effects of aminoguanidine on nitric oxide production induced by inflammatory cytokines and endotoxin in cultured rat hepatocytes. *World J Gastroenterol* 2001; **7**: 331-334
- 21 **Wiest R**, Shah V, Sessa WC, Groszmann RJ. NO overproduction by eNOS precedes hyperdynamic splanchnic circulation in portal hypertensive rats. *Am J Physiol* 1999; **276**(4 Pt 1): G1043-1051
- 22 **Albormoz L**, Bandi JC, de las Heras M, Mastai R. Dexamethasone, an inhibitor of the expression of inducible nitric oxide synthase, does not modify the hyperdynamic state in cirrhotic rats. *Medicina* 2000; **60**: 477-481
- 23 **Chen YM**, Qian ZM, Zhang J, Chang YZ, Duan XL. Distribution of constitutive nitric oxide synthase in the jejunum of adult rat. *World J Gastroenterol* 2002; **8**: 537-539
- 24 **Wolfard A**, Kaszaki J, Szabo C, Balogh Z, Nagy S. Effects of selective nitric oxide synthase inhibition in hyperdynamic endotoxemia in dogs. *Eur Surg Res* 1999; **31**: 314-323
- 25 **Pateron D**, Tazi KA, Sogni P, Heller J, Chagneau C, Poirel O, Philippe M, Moreau R, Lebrec D. Role of aortic nitric oxide synthase 3(eNOS) in the systemic vasodilation of portal hypertension. *Gastroenterology* 2000; **119**: 196-200
- 26 **Villa GL**, Barletta G, Pantaleo P, Bene RD, Vizzutti F, Vecchiarino S, Masini E, Perfetto F, Tarquini R, Gentilini P, Laffi G. Hemodynamic, renal, and endocrine effects of acute inhibition of nitric oxide synthase in compensated cirrhosis. *Hepatology* 2001; **34**: 19-27
- 27 **Kojima H**, Yamao J, Tsujimoto T, Uemura M, Takaya A, Fukui H. Mixed endothelin receptor antagonist, SB209670, decreases portal pressure in biliary cirrhotic rats *in vivo* by reducing portal venous system resistance. *J Hepatol* 2000; **32**: 43-50
- 28 **Kojima H**, Sakurai S, Kuriyama S, Yoshiji H, Imazu H, Uemura M, Nakatani Y, Yamao J, Fukui H. Endothelin-1 plays a major role in portal hypertension of biliary cirrhotic rats through endothelin receptor subtype B together with subtype A *in vivo*. *J Hepatol* 2001; **34**: 805-811
- 29 **Taddei S**, Virdis A, Ghiadoni L, Salvetti A. Vascular effects of endothelin-1 in essential hypertension: relationship with cyclooxygenase-derived endothelium-dependent contracting factors and nitric oxide. *J Cardiovasc Pharmacol* 2000; **35**(4 Suppl 2): S37-40
- 30 **Poo JL**, Jimenez W, Maria Munoz R, Bosch-Marce M, Bordas N, Morales-Ruis M, Perez M, Deulofeu R, Sole M, Arroyo V, Rodes J. Chronic blockade of endothelin receptors in cirrhotic rats: hepatic and hemodynamic effects. *Gastroenterology* 1999; **116**: 161-167
- 31 **Varagic J**, Jerkic M, Jovovic D, Nastic-Miric D, Adanja-Grujic G, Markovic-Lipkovski J, Lackovic V, Radujkovic-Kuburovic G, Kentera D. Regional hemodynamics after chronic nitric oxide inhibition in spontaneously hypertensive rats. *Am J Med Sci* 2000; **320**: 171-176

Edited by Wang XL and Zhu LH

• BASIC RESEARCH •

# Protective effect of taurine on hypochlorous acid toxicity to nuclear nucleoside triphosphatase in isolated nuclei from rat liver

Ju-Xiang Li, Yong-Zheng Pang, Chao-Shu Tang, Zai-Quan Li

**Ju-Xiang Li**, Department of Physiology and Pathophysiology, Health Science Center, Peking University, Beijing 100083, China

**Yong-Zheng Pang, Chao-Shu Tang**, Institute of Cardiovascular Research, First Hospital, Peking University, Beijing 100034, China

**Zai-Quan Li**, Department of Biochemistry and Molecular Biology, Health Science Center, Peking University, Beijing 100083, China

**Supported by** the Major State Basic Research Development Program of People's Republic of China, No. G2000056905 and the National Natural Science Foundation of China, No. 30070308

**Correspondence to:** Zai-Quan Li, Department of Biochemistry and Molecular Biology, Health Science Center, Peking University, Beijing 100083, China. lizaiquan@bjmu.edu.cn

**Telephone:** +86-10-82801631 **Fax:** +86-10-66176255

**Received:** 2003-06-21 **Accepted:** 2003-08-16

## Abstract

**AIM:** Taurine has been shown to be an effective scavenger of hypochlorous acid (HOCl). The role of HOCl is well established in tissue damage associated with inflammation and injury. In the present study, the effect of HOCl on nuclear nucleoside triphosphatase of hepatocytes and the ability of taurine to prevent this effect were investigated.

**METHODS:** Isolated hepatic nuclei from rat liver were exposed to HOCl with or without taurine. The NTPase activity on nuclear envelope was assayed using ATP and GTP as substrates, respectively.

**RESULTS:** The first series of experiments evaluated the toxicity of HOCl and the efficacy of taurine to protect NTPase. HOCl at  $10^{-9}$ - $5 \times 10^{-6}$  mol/L reduced nuclear NTPase activities in a concentration dependent manner (ATP and GTP as substrates) ( $P < 0.01$ ). HOCl at  $10^{-6}$  mol/L reduced the NTPase activity by 65% (ATP as substrate) and 76% (GTP as substrate). Taurine ( $10^{-7}$  to  $10^{-4}$  mol/L) was tested for protection against HOCl at  $10^{-6}$  mol/L and the nuclei treated with  $5 \times 10^{-4}$  mol/L taurine exhibited only 20% and 12% reduction in NTPase activities compared to untreated controls. A second study was performed comparing taurine to glutathione (GSH). GSH and HOCl at  $10^{-6}$  mol/L exhibited 46% and 67.4% reduction in NTPase activities compared with control. GSH ( $10^{-4}$  mol/L) which was incubated with the nuclei and HOCl still exhibited 44.2% and 44.8% reduction in NTPase activities of untreated control. Taurine with HOCl only exhibited 15.2% and 17.1% reduction in NTPase activities, which provided more powerful protection against HOCl than GSH. The third experiment was undertaken to evaluate the specificity of taurine against HOCl. Incubation of rat hepatic nuclei with  $\text{Fe}^{3+}/\text{H}_2\text{O}_2$  (1 m mol/L vs  $5 \mu\text{mol/L}$ ) resulted in a decrease in nuclear NTPase activities ( $P < 0.01$ ). When hepatic nuclei were incubated with Tau ( $10^{-4}$  mol/L) and  $\text{Fe}^{3+}/\text{H}_2\text{O}_2$  (1 m mol/L vs  $5 \mu\text{mol/L}$ ), nuclear NTPase activities were only slightly increased as compared with that of incubation with  $\text{Fe}^{3+}/\text{H}_2\text{O}_2$  alone. However, GSH failed to alter the NTPase activities induced by  $\text{Fe}^{3+}/\text{H}_2\text{O}_2$ .

**CONCLUSION:** The present findings indicate that HOCl

can act as an inhibitor of nuclear NTPase. Taurine can antagonistically reduce the toxicity of HOCl to NTPase.

Li JX, Pang YZ, Tang CS, Li ZQ. Protective effect of taurine on hypochlorous acid toxicity to nuclear nucleoside triphosphatase in isolated nuclei from rat liver. *World J Gastroenterol* 2004; 10 (5): 694-698

<http://www.wjgnet.com/1007-9327/10/694.asp>

## INTRODUCTION

The mechanism of mRNA transport involves two major steps: the recognition of RNA molecules to be transported and their transfer through the nuclear pore. The latter step is an important rate-limiting step in protein expression<sup>[1]</sup>. The nucleocytoplasmic transport of mRNA is an energy-consuming process. The energy requirement is associated with the functioning of a nucleoside triphosphatase (NTPase). The nuclear NTPase activity exhibits a broad substrate specificity toward nucleotides and divalent metal cations<sup>[2,3]</sup>. The recent data demonstrated that the activity of the NTPase was strikingly inhibited by cholesterol oxidase treatment, which indicated that oxidation of nuclear membrane cholesterol could inhibit NTPase activity<sup>[4]</sup>. These results have implications for mRNA flux across the nuclear membrane during conditions where lipid peroxidation may be expected.

Hypochlorous acid (HOCl) is a major oxidant produced by neutrophils and monocytes, via the myeloperoxidase-catalyzed oxidation of chloride by hydrogen peroxide<sup>[5]</sup>. HOCl is a potent oxidant capable of damaging host tissue during inflammation. The strong oxidizing species HOCl plays a highly significant role in the bactericidal function of the neutrophil. However, inappropriate and/or excessive activation of neutrophils leads to oxidative stress and collateral damage to surrounding tissues. Cysteine and methionine residues in proteins and reduced glutathione (GSH) appear to be the main targets for HOCl<sup>[6]</sup>, thereby altering the structure and function of proteins and lowering antioxidant status in the cell. In the literature, taurine, a 2-amino ethanesulfonic acid, is characterized as an antioxidant, a membrane protector, or a regulator of calcium ion homeostasis. It is the major free intracellular amino acid that presents in many tissues<sup>[7,8]</sup> and possibly acts physiologically as a trap for HOCl<sup>[8]</sup>. In the present study, we explored the possible action of HOCl on hepatic nuclear NTPase activity and the protective effect of taurine on the changes of NTPase activity induced by HOCl.

## MATERIALS AND METHODS

### Materials

Male Sprague-Dawley (SD) rats were supplied by the Animal Center, Health-Science Center, Peking University. Taurine and GSH were purchased from Sigma Chemical Co (St. Louis, MO, USA). The term HOCl was used to cover the equilibrium mixture with OCl<sup>-</sup> present at neutral pH. The following reagents were freshly prepared. Phenylmethylsulfonyl fluoride (PMSF), sodium salt of nucleotides (ATP and GTP); DS/PMSF buffer



(mmol/L): 250 sucrose, 50 Tris/HCl pH 7.4, 5 MgCl<sub>2</sub>, 1 PMSF; STM/ Buffer (mmol/L): 2 100 sucrose, 50 Tris/HCl pH 7.4, 5 MgCl<sub>2</sub>, 1 PMSF, 1 EDTA, 1 DTT, and 1  $\mu$ mol/L leupeptin. All the reagents were analytically pure.

### Isolation of rat hepatocytes

Rat hepatocytes were isolated according to Berry and Friend methods<sup>[9]</sup>. Briefly, under anesthesia with urethane (1 g/kg *i.p.*), male SD rats (220–250 g) were in situ liver-perfused at 37 °C via portal vein, with Ca<sup>2+</sup>-free Hanks' solution containing 5 mg/L collagenase and 1 mg · L<sup>-1</sup> hyaluronidase bubbling of 950 mL O<sub>2</sub>-50 mL CO<sub>2</sub>. After 20 min perfusion, the liver was removed, transferred to a beaker containing 200 mL of enzyme medium, broken up with a blunt spatula, and shaken at 37 °C for 15 min in an atmosphere of air. The suspension was filtered through nylon mesh and the cells were separated from debris by centrifuging at 50 g for 2 min. The cells were resuspended in Hanks' solution at 4 °C. Cell viability tested by trypan blue exclusion was higher than 90%.

### Isolation and chracterization of hepatic nuclei

Isolation of rat liver nuclei was performed according to the method described by Kaufmann *et al.*<sup>[10]</sup> with modification. Suspended cells were homogenized in Teflon (10 strokes), sedimented at 800 *r/min* for 10 min. The nuclei were suspended in DS/PMSF buffer, layered over cushions of this buffer, and sedimented at 70 000 *g* for 60 min. Isolated nuclei were resuspended in STM/PMSF buffer, again layered over cushions of DS/PMSF buffer, and sedimented at 70 000 *g* for 30 min. The final pellet was resuspended with STM/PMSF to 1 mg protein/mL, and stored at -70 °C.

Nuclear membrane NADH pyrophosphorylase activity and microsome- NADPH cytochrome-C reductase activity were determined to test the purification of the freshly isolated hepatocyte nuclei.

### Protocol for treatment of isolated nuclei with hypochlorous acid and taurine

Isolated purified nuclei (0.25 mL) were incubated with different chemicals (dissolved in 0.25 mL) for 10 min at 30 °C. The reaction was stopped by cold (4 °C) centrifugation on microcentrifuge for 2 min, and the nuclei pellet was washed once and then resuspended in STM/PMSF to obtain a final protein concentration of 1 mg/mL.

Protocol 1: incubation with buffer alone (control) and sodium hypochlorite (10<sup>-9</sup> to 5×10<sup>-6</sup> mol/L), respectively. Protocol 2: Incubation with buffer alone (control), taurine (10<sup>-6</sup>, 10<sup>-5</sup> and 10<sup>-4</sup> mol/L), sodium hypochlorite (10<sup>-6</sup> mol/L), sodium hypochlorite (10<sup>-6</sup> mol/L) plus taurine (10<sup>-7</sup> to 10<sup>-4</sup> mol/L), respectively. Protocol 3: Incubation with sodium hypochlorite (10<sup>-6</sup> mol/L), sodium hypochlorite (10<sup>-6</sup> mol/L) plus glutathione (GSH, 10<sup>-6</sup> to 10<sup>-4</sup> mol/L), respectively. Protocol 4: Incubation with buffer alone (control), taurine (10<sup>-4</sup> mol/L), GSH (10<sup>-4</sup> mol/L), H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub> (1 mmol/L/5  $\mu$ mol/L), H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub> (1 mmol/L/5  $\mu$ mol/L) plus taurine (10<sup>-6</sup> to 10<sup>-4</sup> mol/L), H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub> (1 mmol/L/5  $\mu$ mol/L) plus GSH (10<sup>-6</sup> to 10<sup>-4</sup> mol/L), respectively.

### Assay of nuclear NTPase activity

NTPase activity was assayed as described by Tiffany<sup>[11]</sup> and Ramjiawan<sup>[12]</sup> with modification. Nuclear suspension (1  $\mu$ g protein/ $\mu$ L) was preincubated for 10 min at 30 °C. Addition of 1.0 mmol/L ATP or 1.0 mmol/L GTP initiated the reaction. Ten minutes after 30 °C-incubation, the reaction was stopped by addition of 100g/L SDS and placing the test tube on ice bath, and inorganic phosphate was measured according to the method of Raess<sup>[13]</sup>, which was expressed as nmol/mgPr per 10 min.

Preliminary experiments showed a linear relationship of NTPase activity with incubation time of nucleoside triphosphate within 30 min. The values were normalized to protein content.

### Data analysis

Separated six experiments were performed in duplicate. All results were expressed as mean±SD. Statistical analysis of the data was performed using one-way analysis of variance followed by Student-Newman-Keuls tests. *P*<0.05 was accepted as statistically significant.

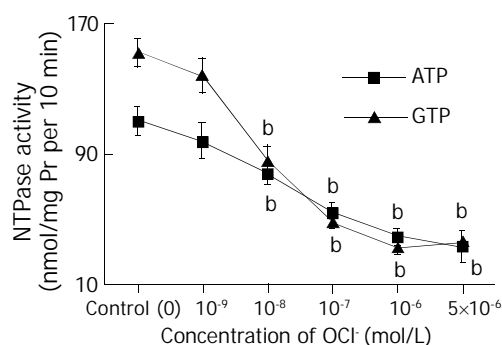
## RESULTS

### Characterization of hepatic nuclei

The level of NADH pyrophosphorylase activity (as marker enzyme for nuclear envelope) in prepared nuclei from rat hepatocytes was 7-fold that in homogenate of whole cells (25.77±1.26 vs 3.68±0.27 nmol/mg Pr per min, *P*<0.01), but NADPH cytochrome C reductase activity (marker enzyme for microsome) was only 28% of that in hypatocytes homogenate (2.88±0.22 vs 10.27±0.87 nmol/mg Pr per min, *P*<0.01). While the activity of mannose-6-phosphatase existing in both microsomes and nuclei, was 4-5 times that in cell homogenate (412±22 vs 91±6 nmol/mg Pr per min, *P*<0.01). It showed that the isolated hepatic nuclear fraction was of high purity and little contaminated by other organelles.

### Inhibitory effect on hepatic nuclear NTPase of hypochlorous acid

HOCl (at mol/L: 10<sup>-9</sup>-5×10<sup>-6</sup>) could significantly depress NTPase activity of hepatic nuclei in a concentration- dependent manner, regardless ATP or GTP as a substrate (Figure 1). After incubation of hepatic nuclei with 5×10<sup>-6</sup> mol · L<sup>-1</sup> HOCl, the hepatic nuclear NTPase activities were decreased by 70.0% (ATP as substrate) and by 76.3% (GTP as substrate), compared with those of control groups (both *P* values less than 0.01) respectively.



**Figure 1** Inhibitory effect of hypochlorous acid on hepatic nuclear NTPase activity. ATP and GTP were used as reaction substrates, respectively. Mean±SD, *n*=6. <sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 compared with control.

### Effects of taurine on hepatic nuclear NTPase activity

The effect of taurine on NTPase activity is shown in Table 1. After incubation of hepatic nuclei with different concentrations of taurine (10<sup>-6</sup>, 10<sup>-5</sup> and 10<sup>-4</sup> mol/L), the NTPase activities on nuclear envelope were increased in a concentration-dependant fashion, either using ATP or GTP as a substrate (all *P* values <0.05 as compared with those of controls). When taurine was at 10<sup>-4</sup> mol/L, the NTPase activities were increased by 18.1% (ATP as substrate) and 27.3% (GTP as substrate), respectively. All *P* values were less than 0.01 as compared with those of their controls.

**Table 1** Effects of taurine on hepatic nuclear NTPase activity

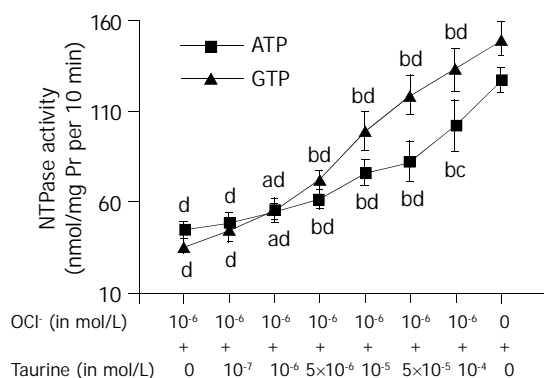
Groups	NTPase activity (nmol/mg Pr per 10 min)	
	ATP as substrate	GTP as substrate
Control	127±7	150±9
Taurine 10 <sup>-6</sup> mol/L	136±9 (+7.1%)	168±10(+12.0%) <sup>a</sup>
Taurine 10 <sup>-5</sup> mol/L	148±7 (+16.5%) <sup>b</sup>	179±11(+19.3%) <sup>b</sup>
Taurine 10 <sup>-4</sup> mol/L	150±8 (+18.1%) <sup>b</sup>	191±12 (±27.3%) <sup>b</sup>

ATP and GTP were used as reaction substrates, respectively. The increases of the enzyme activities are indicated in parentheses as percentage of the control. Mean±SD, *n*=6. <sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 compared with control.

### Effect of taurine on OCl<sup>-</sup>-induced inhibition of hepatic nuclear NTPase activity

The abilities of HOCl to depress NTPase were confirmed by detecting NTPase activities. Incubation of hepatic nuclei with HOCl at 10<sup>-6</sup> mol·L<sup>-1</sup> resulted in an obviously lower nuclear NTPase activity than that with buffer alone. The hepatic nuclear NTPase activities were decreased by 65.4% (ATP as substrate) and by 76.0% (GTP as substrate), compared with the control groups respectively (*P*<0.01).

The reduction of NTPase activities induced by HOCl was antagonized by taurine (as shown in Figure 2), even at a very low concentration (10<sup>-6</sup> mol/L) (ATP and GTP as substrates). The antagonistic effect of taurine on HOCl was in a concentration dependent manner. When the nuclei were incubated with HOCl (10<sup>-6</sup> mol/L) and taurine (5×10<sup>-4</sup> mol/L), the NTPase activity reached 80.3% (ATP as substrate) and 88.7% of control group (GTP as substrate), respectively (all *P* values less than 0.01).

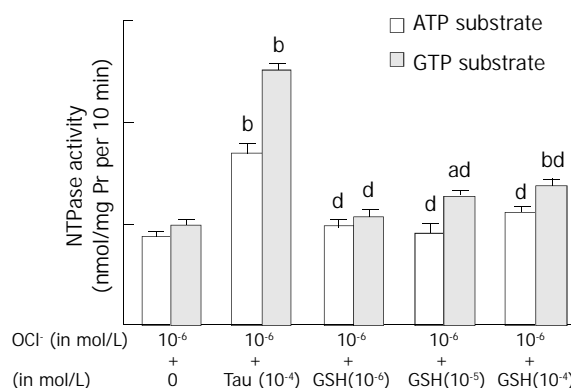


**Figure 2** Effect of taurine on OCl<sup>-</sup>-induced inhibition of NTPase activity in hepatic nuclei. ATP and GTP were used as reaction substrates, respectively. Mean±SD, *n*=6. <sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 compared with OCl<sup>-</sup> (10<sup>-6</sup> mol/L) group. <sup>c</sup>*P*<0.05, <sup>d</sup>*P*<0.01 compared with control.

### Effect of glutathione on OCl<sup>-</sup>-induced inhibition of hepatic nuclear NTPase activity

Incubation of hepatic nuclei with HOCl at 10<sup>-6</sup> mol/L resulted in an obviously lower nuclear NTPase activity. The hepatic nuclear NTPase activities were decreased by 51.2% (ATP as substrate) and by 101.3% (GTP as substrate), compared with the control groups respectively (*P*<0.01). The reduction of NTPase activities induced by HOCl was antagonized by taurine (10<sup>-4</sup> mol/L, ATP and GTP as substrates). Incubation of taurine increased the NTPase activity by 92.6% (ATP as substrate) and 154% (GTP as substrate) compared with HOCl incubation (as shown in Figure 3). GSH incubation attenuated the depressive effect of HOCl in a concentration-dependent manner. When the nuclei were incubated with HOCl (10<sup>-4</sup> mol/L)

and GSH (10<sup>-4</sup> mol/L), the NTPase activity was increased by 27% (ATP as substrate) and 38.5% (GTP as substrate) of HOCl incubation group, respectively (all *P* values less than 0.01). It was showed that the effect of GSH on HOCl-induced depression of NTPase was smaller than that of taurine (*F* value: 5.3, *P*<0.01).



**Figure 3** Effects of glutathione on OCl<sup>-</sup>-induced inhibition of NTPase activity in hepatic nuclei. ATP and GTP were used as reaction substrates, respectively. Mean±SD, *n*=6. <sup>b</sup>*P*<0.01 compared with control group (OCl<sup>-</sup> 10<sup>-6</sup> mol/L). <sup>a</sup>*P*<0.05, <sup>c</sup>*P*<0.01 compared with (10<sup>-6</sup> mol/L OCl<sup>-</sup>+10<sup>-4</sup> mol/L taurine).

### Effect of taurine and GSH on ·OH-induced inhibition of hepatic nuclear NTPase activity

The ability of the Fe<sup>3+</sup>-H<sub>2</sub>O<sub>2</sub> system to produce ·OH was confirmed by detecting NTPase activities. Incubation of hepatic nuclei with Fe<sup>3+</sup>-H<sub>2</sub>O<sub>2</sub> at 1 m mol/L/5 μ mol/L resulted in a lower nuclear NTPase activity as compared with buffer alone. The NTPase activities on nuclear envelopes were decreased by 70% (ATP as substrate) and by 76.7% (GTP as substrate), compared with control group respectively (Table 2).

The reduction of NTPase activities induced by Fe<sup>3+</sup>-H<sub>2</sub>O<sub>2</sub> was antagonized by taurine. When taurine was at 10<sup>-4</sup> mol/L (ATP as substrate), the decreases of NTPase activities induced by Fe<sup>3+</sup>-H<sub>2</sub>O<sub>2</sub> (1 m mol/L/5 μ mol/L) were slightly reversed (from 29.8±8.2 to 46.5±8.7, *P*<0.05). Whereas, GSH, at all concentrations used in our experiment, had no significant effect on Fe<sup>3+</sup>-H<sub>2</sub>O<sub>2</sub>-induced depression of NTPase activity (Table 2).

**Table 2** Effect of taurine and glutathione on ·OH-induced inhibition of NTPase activity in hepatic nuclei

Groups	NTPase activity (nmol/mg Pr per 10 min)	
	ATP as substrate	GTP as substrate
Control	100.0±9.9	151.8±9.9
Tau (10 <sup>-4</sup> mol/L)	138±14.6	175.5±5.9
GSH (10 <sup>-4</sup> mol/L)	95±12.2	150.0±9.8
·OH	29.8±8.2 <sup>b</sup>	35.3±7.8 <sup>b</sup>
·OH+Tau (10 <sup>-6</sup> mol/L)	35.6±6.1	36.2±8.8
·OH+Tau (10 <sup>-5</sup> mol/L)	40.8±8.8	32.7±7.3
·OH+Tau (10 <sup>-4</sup> mol/L)	46.5±8.7 <sup>a</sup>	43.3±7.2
·OH+GSH (10 <sup>-6</sup> mol/L)	32.0±8.2	43.2±11.8
·OH+GSH (10 <sup>-5</sup> mol/L)	32.1±9.7	41.2±9.6
·OH+GSH (10 <sup>-4</sup> mol/L)	44.3±6.2	39.8±5.9

ATP and GTP were used as reaction substrates, respectively. ·OH was produced by Fenton chemistry (Fe<sup>3+</sup>-H<sub>2</sub>O<sub>2</sub>: 1 m mol/L/5 μ mol/L). Mean±SD, *n*=6. Tau: taurine, GSH: glutathione. <sup>b</sup>*P*<0.01 compared with control group (OCl<sup>-</sup> 10<sup>-6</sup> mol·L<sup>-1</sup>). <sup>a</sup>*P*<0.05 compared with ·OH (Fe<sup>3+</sup>-H<sub>2</sub>O<sub>2</sub>: 1 mmol/L/5 μ mol/L) group.

## DISCUSSION

Nuclear NTPase, a nuclear membrane-associated enzyme, provides energy for poly (A)<sup>+</sup>mRNA export through the nuclear pore. Many factors may play a modulatory role in NTPase activity. Extracellular biological active molecules, such as insulin, epidermal growth factor and nuclear membrane cholesterol, could affect NTPase activities through the individual cellular signal transduction system<sup>[14]</sup>. In addition, oxygen derived free radicals of nuclear membrane cholesterol could inhibit nucleoside triphosphatase activity<sup>[4]</sup>. Thus, export of poly (A) mRNA from the nucleus via the nuclear pore complex was influenced, which plays a crucial role in protein synthesis<sup>[1-3, 14]</sup>.

In this present study using nuclei purified from rat hepatocytes, HOCl was confirmed to be a very efficient inhibitor of nuclear NTPase activity. Hepatic nuclear NTPase activity was depressed by incubation of hepatic nuclei with HOCl in a concentration dependent manner, regardless of using ATP or GTP as substrate. It was suggested that NTPase was one of the favorite targets of HOCl. The inhibition of this enzyme might probably be caused by oxidation of an amino acid critical for enzyme function. It is difficult to determine the exact concentration of HOCl that can be reached *in vivo* since it is formed locally and HOCl is very reactive. Concentrations of the drugs in the present study were not quite inadequately used. In our experiments, taurine and GSH were present which might repair the oxidative damage to the NTPase. Therefore the inhibition of nuclear NTPase activity *in vitro* was reversible. Furthermore, taurine has been found to be an activator for nuclear NTPase, since it could stimulate hepatic nuclear NTPase activity in a concentration dependent manner. Taurine and thiol group-containing compounds could play a protecting role during inflammatory processes.

The mechanisms of the effect of HOCl were not concerned in the present studies. It has been shown that HOCl is highly reactive with a wide range of biological molecules<sup>[15,16]</sup>. Of these, thiols are among the most reactive and crucial targets for oxidation in a cell. The deleterious effects of HOCl could be prevented by incubating the nuclei with thiol group-containing compounds as glutathione in the present study. This was in perfect agreement with Pullar *et al*<sup>[6]</sup> who reported that HOCl could react rapidly with thiol groups. The initial product of oxidation of thios by HOCl was sulfenyl chloride<sup>[17]</sup>. It could react with additional thiols to give disulfide<sup>[17]</sup>. Oxidation of sulfhydryl groups in proteins might affect their functional properties. Formation of protein disulfides, mixed disulfides with GSH, or sulfinic acids could result in changes in enzymatic activity, conformation or affinity toward other molecules. Such changes could contribute to the cell damage caused by oxidative stress<sup>[18]</sup>.

As an antioxidant, taurine could effectively antagonize the toxic effect of HOCl on NTPase. However, the mechanism of this effect remains unclear. More recent information has revealed that taurine could interact with peroxide anions to form stable products TauCl<sup>[8]</sup>. The latter was the product formed through the sequestration of taurine with HOCl and has been found to be an exceptionally stable and long-lived compound with cytoprotective properties due to its ability to preserve cellular function in response to physiologic stress<sup>[7]</sup>. In the present study, taurine greatly inhibited the suppression of hepatic nuclear NTPase activity induced by OCl<sup>-</sup>, indicating the important protective role of taurine against OCl<sup>-</sup> attack.

It has been found that oxygen free radical species such as H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub> are produced in mammalian cells during normal aerobic metabolism<sup>[19,20]</sup>. However, O<sub>2</sub> or H<sub>2</sub>O<sub>2</sub> dose not directly act under physiologically relevant conditions. It has been proposed that much of the toxicity of these species in living

organisms be due to the iron-dependent generation of  $\cdot\text{OH}$ , and /or other powerful oxidants, by Fenton chemistry<sup>[21]</sup>. Once it oxidizes Fe<sup>2+</sup>, the reactive  $\cdot\text{OH}$  is produced. Incubation of hepatic nuclei with Fe<sup>3+</sup>-H<sub>2</sub>O<sub>2</sub> in the present study resulted in the decrease of NTPase activities in a concentration dependent manner both using ATP and GTP as substrates, which was coincident with that of Ramjiawan's work<sup>[4]</sup>. The results of this *in vitro* study demonstrated that neither taurine nor GSH could directly prevent the reduction of nuclear NTPase activity caused by the  $\cdot\text{OH}$  producing Fe<sup>3+</sup>/H<sub>2</sub>O<sub>2</sub> system, even if very high concentrations of them (10<sup>-4</sup> mol·L<sup>-1</sup>) were used regardless of using ATP or GTP as substrate. These results therefore suggested that taurine could protect NTPase from HOCl specifically.

It has been found that HOCl is produced under aerobic and pathophysiological conditions such as oxidative stress and inflammation<sup>[22]</sup>. Under most circumstances, HOCl is likely to be the major strong oxidant produced by neutrophils, and contributors to oxidative damages associated with a variety of diseases in which inflammatory cells participate<sup>[23]</sup>. Impairment of NTPase on hepatic nuclei by HOCl might result in default of RNA nucleocytoplasmic transport. Taurine could antagonize the toxic effect of HOCl on NTPase. This observation could be a part of the global machinery, which acts as a cytoprotective factor in liver inflammation and oxygen stress.

In summary, our results showed that HOCl could cause a decrease in nuclear NTPase activities, which was most likely the result of decreased breakdown of NTPase. This pointed toward HOCl as an inhibitor of this enzyme. Nuclear NTPase can be effectively protected by taurine against HOCl driven oxidative injury, a consequence of direct drug scavenging capacity towards HOCl. Interaction of taurine with HOCl can also protect nuclear NTPase activity. Therefore, taurine treatment would have a beneficial effect on some diseases relating to protein synthesis.

## REFERENCES

- 1 **Izaurrealde E**, Mattaj IW. RNA export. *Cell* 1995; **81**: 153-159
- 2 **Tomassoni ML**, Amori D, Magni MV. Changes of nuclear membrane lipid composition affect RNA nucleocytoplasmic transport. *Biochem Biophys Res Commun* 1999; **258**: 476-481
- 3 **Agutter PS**. Influence of nucleotides, cations and nucleoside triphosphatase inhibitors on the release of ribonucleic acid from isolated rat liver nuclei. *Biochem J* 1980; **188**: 91-97
- 4 **Ramjiawan B**, Czubryt MP, Massaeli H, Gilchrist JS, Pierce GN. Oxidation of nuclear membrane cholesterol inhibits nucleoside triphosphatase activity. *Free Radic Biol Med* 1997; **23**: 556-562
- 5 **Winterbourn CC**, Vissers MC, Kettle AJ. Myeloperoxidase. *Curr Opin Hematol* 2000; **7**: 53-58
- 6 **Pullar JM**, Winterbourn CC, Vissers MC. Loss of GSH and thiol enzymes in endothelial cells exposed to sublethal concentrations of hypochlorous acid. *Am J Physiol* 1999; **277**(4 Pt 2): H1505-1512
- 7 **Lourenco R**, Camilo ME. Taurine: a conditionally essential amino acid in humans? An overview in health and disease. *Nutr Hosp* 2002; **17**: 262-270
- 8 **Huxtable RJ**. Physiological actions of taurine. *Physiol Rev* 1992; **72**: 101-163
- 9 **Berry MN**, Friend DS. High-yield preparation of isolated rat liver parenchymal cells. *J Cell Biol* 1969; **43**: 506-520
- 10 **Kaufmann SH**, Gibson W, Shaper JH. Characterization of the major polypeptides of the rat liver nuclear envelope. *J Biol Chem* 1983; **258**: 2710-2719
- 11 **Tiffany BR**, White BC, Krause GS. Nuclear-envelope nucleoside triphosphatase kinetics and mRNA transport following brain ischemia and reperfusion. *Ann Emerg Med* 1995; **25**: 809-817
- 12 **Ramjiawan B**, Czubryt MP, Gilchrist JS, Pierce GN. Nuclear membrane cholesterol can modulate nuclear nucleoside triphosphatase

- activity. *J Cell Biochem* 1996; **63**: 442-452
- 13 **Racess BU**, Vincenzi FF. A semi-automated method for the determination of multiple membrane ATPase activities. *J Pharmacol Methods* 1980; **4**: 273-283
- 14 **Schroder HC**, Wenger R, Ugarkovic D, Friese K, Bachmann M, Muller WE. Differential effect of insulin and epidermal growth factor on the mRNA translocation system and transport of specific poly (A+) mRNA and poly (A-) mRNA in isolated nuclei. *Biochemistry* 1990; **29**: 2368-2378
- 15 **Prutz WA**. Hypochlorous acid interactions with thiols, nucleotides, DNA, and other biological substrates. *Arch Biochem Biophys* 1996; **332**: 110-120
- 16 **Prutz WA**, Kissner R, Koppenol WH, Ruegger H. On the irreversible destruction of reduced nicotinamide nucleotides by hypohalous acids. *Arch Biochem Biophys* 2000; **380**: 181-191
- 17 **Peskin AV**, Winterbourn CC. Kinetics of the reactions of hypochlorous acid and amino acid chloramines with thiols, methionine, and ascorbate. *Free Radic Biol Med* 2001; **30**: 572-579
- 18 **Sen CK**, Packer L. Antioxidant and redox regulation of gene transcription. *FASEB J* 1996; **10**: 709-720
- 19 **Jaeschke H**. Mechanisms of oxidant stress-induced acute tissue injury. *Proc Soc Exp Biol Med* 1995; **209**: 104-111
- 20 **Simpkins CO**. Metallothionein in human disease. *Cell Mol Biol* 2000; **46**: 465-488
- 21 **Halliwell B**, Gutteridge JM. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* 1990; **186**: 1-85
- 22 **Bomzon A**, Ljubuncic P. Oxidative stress and vascular smooth muscle cell function in liverd isease. *Pharmacol Ther* 2001; **89**: 295-308
- 23 **Winterbourn CC**, Kettle AJ. Biomarkers of myeloperoxidase-derived hypochlorous acid. *Free Radic Biol Med* 2000; **29**: 403-409

Edited by Wang XL Proofread by Zhu LH

# Primary hepatocyte culture in collagen gel mixture and collagen sandwich

Ying-Jie Wang, Hong-Ling Liu, Hai-Tao Guo, Hong-Wei Wen, Jun Liu

**Ying-Jie Wang, Hong-Ling Liu, Hai-Tao Guo, Hong-Wei Wen, Jun Liu**, Institute of Infectious Diseases, Southwest Hospital, Third Military Medical University, Chongqing 400038, China  
**Supported by** the National Natural Science Foundation of China, No. 30027001

**Correspondence to:** Dr. Ying-Jie Wang, Institute of Infectious Diseases, Southwest Hospital, Third Military Medical University, Chongqing 400038, China. wangyj103@263.net  
**Telephone:** +86-23-68754479-8062  
**Received:** 2003-08-05 **Accepted:** 2003-09-24

## Abstract

**AIM:** To explore the methods of hepatocytes culture in a collagen gel mixture or between double layers of collagen sandwich configuration and to examine the functional and cytomorphological characteristics of cultured hepatocytes.

**METHODS:** A two-step collagenase perfusion technique was used to isolate the hepatocytes from Wistar rats or newborn Chinese experimental piglets. The isolated hepatocytes were cultured in a collagen gel mixture or between double layers of collagen sandwich configuration respectively. The former was that rat hepatocytes were mixed with type I rat tail collagen solution till gelled, and the medium was added onto the gel. The latter was that swine hepatocytes were seeded on a plate precoated with collagen gel for 24 h, then another layer of collagen gel was overlaid, resulting in a sandwich configuration. The cytomorphological characteristics, albumin secretion, and LDH-release of the hepatocytes cultured in these two models were examined.

**RESULTS:** Freshly isolated rat hepatocytes were successfully mixed and fixed in collagen gel, and cultured in the gel condition. During the culture period, the urea synthesized and secreted by rat hepatocytes was detected throughout the period. Likewise, newborn experimental piglet hepatocytes were successfully fixed between the double layers of collagen gel, forming a sandwich configuration. Within a week of culture, the albumin secreted by swine hepatocytes was detected by SDS/PAGE analysis. The typical cytomorphological characteristics of the hepatocytes cultured by the above two culture models were found under a phase-contrast microscope. There was little LDH-release during the culture period.

**CONCLUSION:** Both collagen gel mixture and double layers of collagen sandwich configuration can provide cultural conditions much closer to *in vivo* environment, and are helpful for maintaining specific hepatic functions and cytomorphological characteristics. A collagen gel mixture culture may be more eligible for the study of bioartificial livers.

Wang YJ, Liu HL, Guo HT, Wen HW, Liu J. Primary hepatocyte culture in collagen gel mixture and collagen sandwich. *World J Gastroenterol* 2004; 10(5): 699-702  
<http://www.wjgnet.com/1007-9327/10/699.asp>

## INTRODUCTION

Isolated and cultured hepatocytes *in vitro* are an important tool for hepatic disease study, and have been extensively used in basic researches of liver disease, pathophysiology, pharmacology and other related subjects<sup>[1-5]</sup>. Furthermore, they are a core material of bioartificial liver system which has been developed rapidly in recent years<sup>[6-10]</sup>. However, normal culture condition of hepatocytes *in vitro* differs greatly from the environment *in vivo*, and is difficult to maintain the physiological function of hepatocytes, leading to restriction of their extensive uses. Along with the advances in the research of bioartificial liver support system and its related fields, a highly active hepatocyte culture system is urgently required to meet the quality requirement of cultured hepatocytes in these studies<sup>[11-15]</sup>.

It has been discovered that collagen is one kind of important matrix of hepatocytic basal membrane. Type I rat tail collagen is usually used as a coated material for hepatocyte culture. It promotes attachment and growth of hepatocytes. The effect of culture is much better than that without collagen coating, indicating that collagen matrix might be an important condition for long-term hepatocyte culture<sup>[16-18]</sup>. Based on this idea, we performed the collagen mixture culture for rat hepatocytes and the collagen sandwich culture for new-born Chinese experimental piglet hepatocytes, in order to improve the effect of hepatocyte culture, and provide experimental data for bioartificial liver.

## MATERIALS AND METHODS

### Animal

Wistar rats (both sexes, less than one month old) and newborn Chinese experimental piglets (both sexes, one week old) were provided by the Experimental Animal Center, Third Military Medical University. All studies were performed with the approval of Experimental Animal Committee in our university. The animals were fed under standard conditions and fasted for 4 h before the experiment.

### Reagents

Type IV collagenase, type I mouse collagen (collagen VII) and epidermal growth factor (EGF) were purchased from Sigma. DMEM medium, RPMI1640 medium and FBS were from Gibco. Acrylamide, N,N'-methylene-bis-acrylamide, sodium dodecyl sulfate (SDS), Coomassie blue were from Shengong Biothech Company (Shanghai, China).

### Cell isolation

Hepatocytes were harvested using a two-step collagenase perfusion technique described previously<sup>[19,20]</sup>. Briefly, an animal was anesthetized by pentobarbital sodium (30 mg/kg), followed by sterilizing the skin, opening the abdomen, exposing the portal vein, and heparin sodium (100-150 u) was injected into the portal vein. When the liver was isolated at liver hilus, it was first perfused with calcium and magnesium-free Hank's buffer at 80-100 mL/min for 10-15 min. The liver was then perfused with 0.5g/L collagenase solution at 50-70 mL/min for

10 min. The two perfusion systems were kept at 37-38 °C. After perfusion, the liver capsule was incised. The thick fibrous connective tissue was discarded, and cell suspensions were harvested. The cell suspensions were further digested at 37 °C for 10-15 min in case. When RPMI 1640 medium was used for cessation of digestion, the released cells were filtered through three-layer sterilized gauze and washed via three centrifugations (50 g). The hepatocyte viability greater than 95% as determined by trypan blue exclusion was used for culture.

#### **Collagen mixture gel culture**

The collagen mixture consisted of a 4:1 ratio of rats tail collagen and 4× DMEM brought to a pH of 7.2 using 1mol/L NaOH.  $1 \times 10^6$  isolated rat hepatocytes were mixed with collagen solution and plated in a flask. The height of hepatocyte-collagen mixture was 0.3-0.4 cm. The mixture was incubated in a 37 °C incubator for 2 h until the collagen mixture was gelled, resulting in hepatocyte-collagen mixture gel. After washed 2-3 times with PBS, RPMI1640 medium containing 100mL/L FBS and EFG was added and the cells were cultured in normal conditions. The medium was changed daily and the culture was over on day 9. The normal single layer culture was used as control, the seeding density and medium were the same as collagen gel mixture culture.

#### **Collagen sandwich culture**

The collagen solution was prepared as described above, and coated on a 6-well plate. Collagen solution (1.2 mL) was added into each well and incubated in a 37 °C incubator for 2 h until the collagen was gelled. Freshly isolated hepatocytes ( $10^6$ ) were plated on a 6-well plate precoated with collagen gel and cultured with serum free RPMI 1640. After 24 h, the culture medium was replaced by 1.2 mL/well collagen solution, and incubated in an incubator for 1 h until the collagen was fixed. The hepatocytes were cultured under the above conditions. The medium was changed daily and the culture was over on day 7.

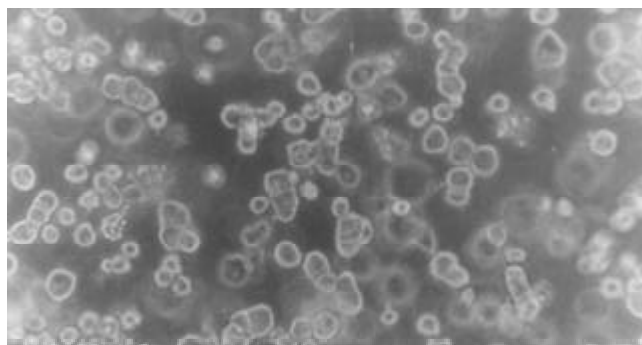
#### **Morphological and functional observations of cultured hepatocytes**

Supernatant of the medium was collected every day during the rat hepatocytes-collagen gel culture, and BUN was measured using a biochemical autoanalyzer (Model 7020, Hitach Co., Tokyo, Japan). Twenty-five  $\mu$ L of the supernatant of serum free medium with piglet hepatocyte sandwich culture was harvested on days 1, 2, 5, and 7 respectively, and separated on a 120 g/L SDS-PAGE, resulting in porcine albumin bands. The gel was stained with 5g/L Coomassie blue, and the images were analyzed with the gel documentation-analyzing systems (Gel DocTM 2000, Bio-Rad, USA). LDH-release in the supernatants of the two kinds of hepatocyte culture media was analyzed by ultraviolet dynamic method. Morphological changes of hepatocytes in both collagen gel mixture and sandwich cultures were observed under a phase-contrast microscope.

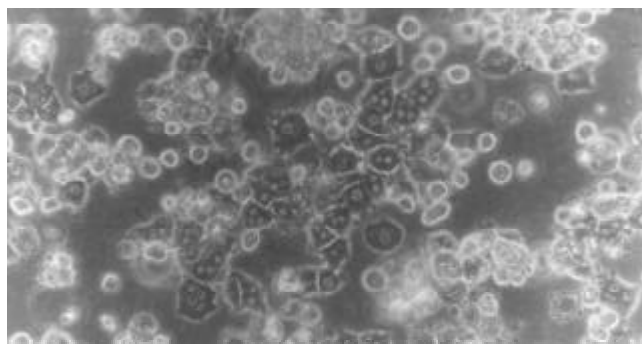
### **RESULTS**

**Morphology of rat hepatocytes cultured in collagen gel mixture**  
When freshly isolated hepatocytes were mixed with collagen and seeded on the culture plate, spherical hepatocytes were mixed in a slightly transparent collagen solution. When the collagen solution was gelled, the hepatocytes were uniformly fixed within. After cultured for 24 h, most hepatocytes remained spherical in shape with part of the cells transformed into polygonal shape. The majority of hepatocytes adhered to each other presenting a fascicular growth (Figure 1). After cultured for 48 h, the majority of hepatocytes reconstructed their cellular polarity presenting a typical and even polygonal

morphology. Binuclei were observed in the majority of hepatocytes, the hepatic plate structure was gradually formed, and the boundary between hepatocytes was perfectly clear and bright, illustrating the formation and participation of bile canaliculi (Figure 2). Such cytomorphological characteristics remained till the end of culture. The rat hepatocytes via an ordinary single layer culture quickly sank and attached to the bottom of culture plate after seeding. Their cytomorphology changed from freshly isolated spherical morphology to a monolayer flat polygon. The cells became flat 48-72 h later and gradually lost their typical polygonal characteristic, and eventually, the hepatocytes were in conjunction with each other and grew in patches.



**Figure 1** Morphology of rat hepatocytes in collagen gel mixture culture at 24 h (phase-contrast microscope  $\times 200$ ).



**Figure 2** Morphology of rat hepatocytes in collagen gel mixture culture at 72 h (phase-contrast microscope  $\times 200$ ).

#### **Morphological changes of hepatocytes cultured in sandwich configurations**

After freshly isolated piglet hepatocytes were seeded on a single layer of collagen gel, the hepatocytes soon attached to the culture plates and their spherical morphology changed quickly to polygonal morphology. After 4 h of culture, the hepatocytes exhibited colonial and fascicular growth, showing typical, uniform polygonal morphology (Figure 3).

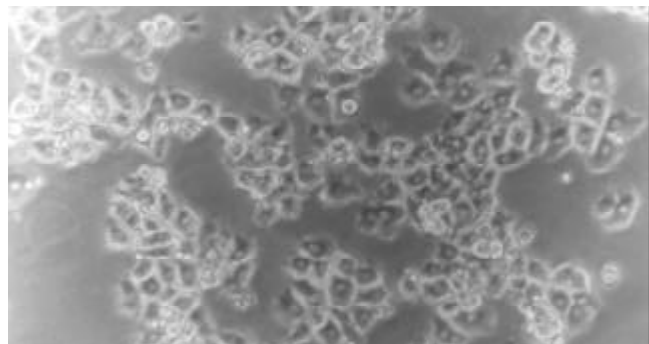
Twenty-four hours after overlaying the upper-layer of the gel, the cells still remained polygonal in shape. Over the culture period, polygonal characteristics were much more typical, and showed unique nuclei, most of which were binuclei. Another unique appearance was that the cellular border was clear and bright among the cells, indicating formation of bile canaliculi. The reconstructed hepatocytes had a cellular polarity and a plate-like structure, and maintained their characteristics till the end of culture (Figure 4).

#### **Urea synthesis by rat hepatocytes cultured in collagen gel mixture**

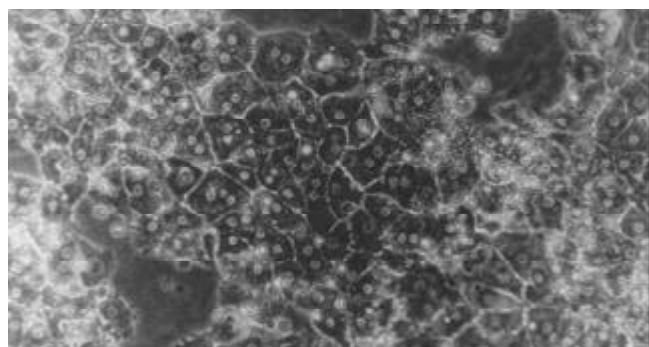
Figure 5 shows the BUN level in supernatant of the medium



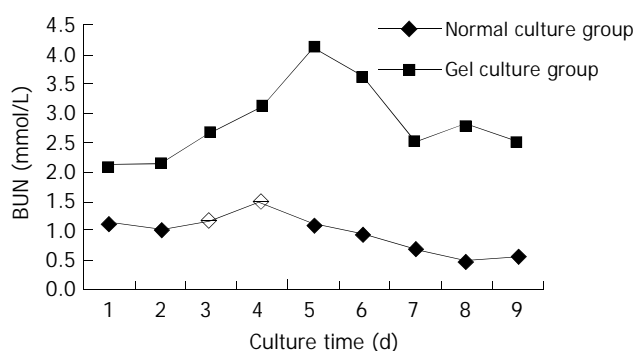
by dynamic measurement. The BUN level in collagen gel mixture culture group was higher than that in the normal culture group throughout the period, and was significantly higher on d 3, 4, 5, 7 and 8 ( $n=5$ ,  $P<0.05$ ).



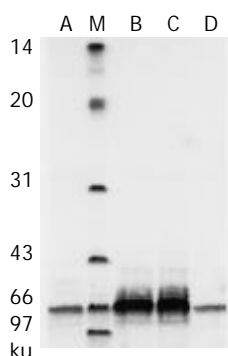
**Figure 3** Morphology of piglet hepatocytes cultured on a single layer of collagen gel at 4 h (phase-contrast microscope  $\times 200$ ).



**Figure 4** Morphology of piglet hepatocytes cultured in sandwich configurations on d 5 (phase-contrast microscope  $\times 200$ ).



**Figure 5** BUN level in supernatant of medium.



**Figure 6** Albumin secretion in cultured piglet hepatocytes by SDS-PAGE analysis. M: Low-range protein molecular weight markers; lanes A, B, C, D are culture media on days 1, 3, 5, 7.

### Albumin secretion of piglet hepatocytes

Porcine albumin released from piglet hepatocytes was detected by SDS-PAGE during the procedure of sandwich culture and high rates of urea biosynthesis were maintained throughout this period (Figure 6).

### LDH-release

The amount of LDH released from hepatocytes cultured in collagen gel was detected to be an average of  $106 \pm 17.4$  U/L by dynamic measurement in supernatant of the medium. The amount of LDH in supernatant of the medium with sandwich culture fluctuated between 24.8–58.6 U/L, and was not significantly different ( $P>0.05$ ) at various batches. LDH release was declined both in collagen gel mixture and in sandwich culture and remained at a similar low level towards the end of culture.

### DISCUSSION

Along with the improvement in the techniques of enzyme-digested hepatocyte isolation in recent years, hepatocyte culture has been applied extensively. However, since the complexity of liver structure and function as well as the existence of specificity of hepatocytes *in vivo*, isolating hepatocytes *in vitro* requires very strict conditions. Monolayer attachment culture model is not suitable for hepatocytes *in vivo*, it only meets the general experimental requirements so far. In order to overcome this difficulty, enormous researches have been carried out to improve the media, supplements, growth factors, matrix or culture methods in hepatocyte culture. A series of methods have been developed, such as specified medium, addition of trace elements, transferrin, insulin, HGF, EGF, coated specific matrix, and the use of microcarrier as well as mixed culture<sup>[21–28]</sup>. However, a mature technique meeting the specific requirements of research in bioartificial liver supporting system requires a large scale of high density, high activity, and long-term hepatocyte culture. This has not been developed so far. Therefore, the exploration of hepatocyte culture with high activity becomes an important subject in this research field.

It is known that normal hepatocytes appear as a three dimensional structure arranged in the liver lobules. There exists a complicated connection between hepatocytes, parenchymal-nonparenchymal cells, and hepatocytes-extracellular matrix. Among them, the matrix has been found to play an important role in maintaining hepatocytic functions. A large number of researches have shown that using collagen to fix hepatocytes in the manner of sandwich configuration could create a matrix environment close to that *in vivo*, and availed thus, hepatocytes of growth and their specific function. In this study, freshly isolated hepatocytes from newborn Chinese experimental piglets were cultured between double layers of collagen sandwich configuration, and the hepatocytes were found to have good functions of protein secretion and maintained their cytomorphological characteristics with little LDH-leakage. This illustrated that double layers of collagen sandwich configuration could provide a fairly good cultural and growth condition to piglet hepatocytes with high activity. Even though the double layers of collagen sandwich configuration technique could be used in highly active hepatocyte culture, it is still a monolayer attachment culture, the liver plate so formed is still unlike the normal three dimensional structure of hepatocytes *in vivo*, and also lacks connections with other kinds of cells in the liver. In addition, the cell density is not high, and operation is not convenient, requiring digestion twice. Thereby, the double layers of collagen sandwich technique might not be suitable for the study of bioartificial liver supporting system and hepatocyte transplantation.

Collagen gel mixture is a novel gel immobilizing technique. In this method, the hepatocytes, collagen and medium are mixed and gelled after seeding. The medium was added on its top. Our study showed that rat hepatocytes cultured by such a method could uniformly mix in collagen gel, showing high-density and a three dimensional structural characteristic. During the culture period, the urea synthesizing ability of rat hepatocytes cultured by collagen gel mixture was obviously superior to that of hepatocytes cultured ordinarily and the LDH-release reflecting the hepatocyte activity was close to that of hepatocytes cultured ordinarily. This illustrated that collagen gel mixture could provide fine hepatocytes culture and growth with high density and high activity. The greatest advantage of this culture method is that the structure of cultured hepatocytes is closer to the normal three dimensional hepatocyte structure *in vivo* with the characteristics of high density and more extensive connections between hepatocytes and other cells. Therefore, collagen gel mixture immobilization may become one of the effective methods for hepatocytes culture in the bioartificial liver supporting system.

## REFERENCES

- 1 **Low-Baselli A**, Hufnagl K, Parzefall W, Schulte-Hermann R, Grasl-Kraupp B. Initiated rat hepatocytes in primary culture: a novel tool to study alterations in growth control during the first stage of carcinogenesis. *Carcinogenesis* 2000; **21**: 79-86
- 2 **Hoebe KH**, Witkamp RF, Fink-Gremmels J, Van Miert AS, Monshouwer M. Direct cell-to-cell contact between Kupffer cells and hepatocytes augments endotoxin-induced hepatic injury. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G720-728
- 3 **Yao XX**, Tang YW, Yao DM, Xiu HM. Effects of Yigan Decoction on proliferation and apoptosis of hepatic stellate cells. *World J Gastroenterol* 2002; **8**: 511-514
- 4 **Delmas J**, Schorr O, Jamard C, Gibbs C, Trepo C, Hantz O, Zoulim F. Inhibitory effect of adefovir on viral DNA synthesis and covalently closed circular DNA formation in duck hepatitis B virus-infected hepatocytes *in vivo* and *in vitro*. *Antimicrob Agents Chemother* 2002; **46**: 425-433
- 5 **Martin-Aragon S**, de las Heras B, Sanchez-Reus MI, Benedi J. Pharmacological modification of endogenous antioxidant enzymes by ursolic acid on tetrachloride-induced liver damage in rats and primary cultures of rat hepatocytes. *Exp Toxicol Pathol* 2001; **53**: 199-206
- 6 **Donato MT**, Castell JV, Gomez-Lechon MJ. Characterization of drug metabolizing activities in pig hepatocytes for use in bioartificial liver devices: comparison with other hepatic cellular models. *J Hepatol* 1999; **31**: 542-549
- 7 **Gerlach JC**, Zeilinger K, Sauer IM, Mieder T, Naumann G, Grunwald A, Pless G, Holland G, Mas A, Vienken J, Neuhaus P. Extracorporeal liver support: porcine or human cell based systems? *Int J Artif Organs* 2002; **25**: 1013-1018
- 8 **Sauer IM**, Zeilinger K, Obermayer N, Pless G, Grunwald A, Pascher A, Mieder T, Roth S, Goetz M, Kardassis D, Mas A, Neuhaus P, Gerlach JC. Primary human liver cells as source for modular extracorporeal liver support—a preliminary report. *Int J Artif Organs* 2002; **25**: 1001-1005
- 9 **Morsiani E**, Brogli M, Galavotti D, Pazzi P, Puviani AC, Azzena GF. Biologic liver support: optimal cell source and mass. *Int J Artif Organs* 2002; **25**: 985-993
- 10 **Vilei MT**, Granato A, Ferraresso C, Neri D, Carraro P, Gerunda G, Muraca M. Comparison of pig, human and rat hepatocytes as a source of liver specific metabolic functions in culture systems—implications for use in bioartificial liver devices. *Int J Artif Organs* 2001; **24**: 392-396
- 11 **Linti C**, Zipfel A, Schenk M, Dauner M, Doser M, Viebahn R, Becker HD, Planck H. Cultivation of porcine hepatocytes in polyurethane nonwovens as part of a biohybrid liver support system. *Int J Artif Organs* 2002; **25**: 994-1000
- 12 **Jasmund I**, Langsch A, Simmoteit R, Bader A. Cultivation of primary porcine hepatocytes in an OXY-HFB for use as a bioartificial liver device. *Biotechnol Prog* 2002; **18**: 839-846
- 13 **Nakazawa K**, Ijima H, Fukuda J, Sakiyama R, Yamashita Y, Shimada M, Shirabe K, Tsujita E, Sugimachi K, Funatsu K. Development of a hybrid artificial liver using polyurethane foam/hepatocyte spheroid culture in a preclinical pig experiment. *Int J Artif Organs* 2002; **25**: 51-60
- 14 **Yamashita Y**, Shimada M, Tsujita E, Tanaka S, Ijima H, Nakazawa K, Sakiyama R, Fukuda J, Ueda T, Funatsu K, Sugimachi K. Polyurethane foam/spheroid culture system using human hepatoblastoma cell line (Hep G2) as a possible new hybrid artificial liver. *Cell Transplant* 2001; **10**: 717-722
- 15 **Yamashita Y**, Shimada M, Tsujita E, Rikimaru T, Ijima H, Nakazawa K, Sakiyama R, Fukuda J, Funatsu K, Sugimachi K. The efficacy of nafamostat mesilate on the performance of a hybrid-artificial liver using a polyurethane foam/porcine hepatocyte spheroid culture system in human plasma. *Int J Artif Organs* 2001; **24**: 34-40
- 16 **Nagaki M**, Miki K, Kim YI, Ishiyama H, Hirahara I, Takahashi H, Sugiyama A, Muto Y, Moriwaki H. Development and characterization of a hybrid bioartificial liver using primary hepatocytes entrapped in a basement membrane matrix. *Dig Dis Sci* 2001; **46**: 1046-1056
- 17 **Luttringer O**, Theil FP, Lave T, Wernli-Kuratli K, Guentert TW, de Saizieu A. Influence of isolation procedure, extracellular matrix and dexamethasone on the regulation of membrane transporters gene expression in rat hepatocytes. *Biochem Pharmacol* 2002; **64**: 1637-1650
- 18 **Hong JT**, Glauert HP. Effect of extracellular matrix on the expression of peroxisome proliferation associated genes in cultured rat hepatocytes. *Toxicol In Vitro* 2000; **14**: 177-184
- 19 **Wang YJ**, Li MD, Wang YM, Chen GZ, Lu GD, Tan ZX. Effect of extracorporeal bioartificial liver support system on fulminant hepatic failure rabbits. *World J Gastroenterol* 2000; **6**: 252-254
- 20 **Wang YJ**, Li MD, Wang YM, Nie QH, Chen GZ. Experimental study of bioartificial liver with cultured human liver cells. *World J Gastroenterol* 1999; **5**: 135-137
- 21 **Zeilinger K**, Sauer IM, Pless G, Strobel C, Rudzitis J, Wang A, Nussler AK, Grebe A, Mao L, Auth SH, Unger J, Neuhaus P, Gerlach JC. Three-dimensional co-culture of primary human liver cells in bioreactors for *in vitro* drug studies: effects of the initial cell quality on the long-term maintenance of hepatocyte-specific functions. *Altern Lab Anim* 2002; **30**: 525-538
- 22 **Sakai Y**, Jiang J, Kojima N, Kinoshita T, Miyajima A. Enhanced *in vitro* maturation of fetal mouse liver cells with oncostatin M, nicotinamide, and dimethyl sulfoxide. *Cell Transplant* 2002; **11**: 435-441
- 23 **Klein H**, Ullmann S, Drenckhan M, Grimmsmann T, Unthan-Fechner K, Probst I. Differential modulation of insulin actions by dexamethasone: studies in primary cultures of adult rat hepatocytes. *J Hepatol* 2002; **37**: 432-440
- 24 **Yamashita Y**, Shimada M, Tsujita E, Shirabe K, Ijima H, Nakazawa K, Sakiyama R, Fukuda J, Funatsu K, Sugimachi K. High metabolic function of primary human and porcine hepatocytes in a polyurethane foam/spheroid culture system in plasma from patients with fulminant hepatic failure. *Cell Transplant* 2002; **11**: 379-384
- 25 **Katsura N**, Ikai I, Mitaka T, Shiotani T, Yamanokuchi S, Sugimoto S, Kanazawa A, Terajima H, Mochizuki Y, Yamaoka Y. Long-term culture of primary human hepatocytes with preservation of proliferative capacity and differentiated functions. *J Surg Res* 2002; **106**: 115-123
- 26 **Kamiya A**, Kojima N, Kinoshita T, Sakai Y, Miyajima A. Maturation of fetal hepatocytes *in vitro* by extracellular matrices and oncostatin M: induction of tryptophan oxygenase. *Hepatology* 2002; **35**: 1351-1359
- 27 **Wang YJ**, Li MD, Wang YM, Ding J, Nie QH. Simplified isolation and spheroidal aggregate culture of rat hepatocytes. *World J Gastroenterol* 1998; **4**: 74-76
- 28 **Washizu J**, Berthiaume F, Chan C, Tompkins RG, Toner M, Yarmush ML. Optimization of rat hepatocyte culture in citrated human plasma. *J Surg Res* 2000; **93**: 237-246

# Therapeutic effects and molecular mechanisms of anti-fibrosis herbs and selenium on rats with hepatic fibrosis

Yu-Tong He, Dian-Wu Liu, Li-Yu Ding, Qing Li, Yong-Hong Xiao

**Yu-Tong He, Dian-Wu Liu, Li-Yu Ding, Qing Li, Yong-Hong Xiao**, Department of Epidemiology, Hebei Medical University, Shijiazhuang 050017, Hebei Province, China

**Supported by** the Natural Science Foundation of Hebei Province, No. 302489

**Correspondence to:** Dr. Dian-Wu Liu, Department of Epidemiology, Hebei Medical University, Shijiazhuang 050017, Hebei Province, China. liudianw@hebmu.edu.cn

**Telephone:** +86-311-6265601 **Fax:** +86-311-6265531

**Received:** 2003-06-05 **Accepted:** 2003-10-12

## Abstract

**AIM:** To study the therapeutic effects of anti-fibrosis herbs and selenium on hepatic fibrosis induced by carbon tetrachloride (CCl<sub>4</sub>) in rats and the underlining molecular mechanisms.

**METHODS:** Fifty-three Wistar rats were randomly divided into: normal control group, model control group, colchicine group, anti-fibrosis herbs group (AF group) and anti-fibrosis herbs plus selenium group (AS group). The last four groups were administered with CCl<sub>4</sub> at the beginning of experiment to induce hepatic fibrosis. Then colchicine, anti-fibrosis herbs and selenium were used to treat them. The normal control group and the model control group were given normal saline at the same time. At the end of the 6<sup>th</sup> week, rats in each group were sacrificed. Blood and tissue specimens were taken. Serum indicators (ALT, AST, HA, LN) were determined and histopathological changes were graded. Lymphocyte CD<sub>4</sub> and CD<sub>8</sub> were examined by flow cytometry. Expression of TGF- $\beta$ <sub>1</sub> and NF- $\kappa$ B was detected by immunohistochemistry and expression of TGF- $\beta$ <sub>1</sub> mRNA was detected by semi-quantified RT-PCR.

**RESULTS:** Histological grading showed much a smaller degree of hepatic fibrogenesis in AS group and AF group than that in colchicine group and model control group. The serum content of ALT, AST, HA and LN in AF group and AS group were significantly lower than that in colchicine group (ALT: 65.8 $\pm$ 26.5, 67.3 $\pm$ 18.4 and 96.2 $\pm$ 20.9 in AF, AS and colchicine groups respectively; AST: 150.8 $\pm$ 34.0, 154.6 $\pm$ 27.3 and 215.8 $\pm$ 24.6 respectively; HA: 228 $\pm$ 83, 216 $\pm$ 58 and 416 $\pm$ 135 respectively; LN: 85.9 $\pm$ 15.0, 80.6 $\pm$ 18.6 and 106.3 $\pm$ 14.2 respectively) ( $P$ <0.05). The level of CD<sub>4</sub> and CD<sub>4</sub>/CD<sub>8</sub> ratio in AF group and AS group was significantly higher than those in colchicine group (CD<sub>4</sub>: 50.8 $\pm$ 3.8, 52.6 $\pm$ 3.4 and 40.2 $\pm$ 2.1 in AF, AS and colchicine groups respectively; CD<sub>4</sub>/CD<sub>8</sub> ratio: 1.45, 1.46 and 1.26, respectively) ( $P$ <0.05). The expression level of NF- $\kappa$ B and TGF- $\beta$ <sub>1</sub> in the liver tissues of AF and AS treatment groups was markedly decreased compared with that in colchicine group, and TGF- $\beta$ <sub>1</sub> mRNA was also markedly decreased (1.07 $\pm$ 0.31 and 0.98 $\pm$ 0.14 vs 2.34 $\pm$ 0.43,  $P$ <0.05).

**CONCLUSION:** Anti-fibrosis herbs and selenium have beneficial effects on hepatic fibrosis in rats by enhancing immunity and inhibiting NF- $\kappa$ B and TGF- $\beta$ <sub>1</sub> expressions.

He YT, Liu DW, Ding LY, Li Q, Xiao YH. Therapeutic effects and molecular mechanisms of anti-fibrosis herbs and selenium on rats with hepatic fibrosis. *World J Gastroenterol* 2004; 10 (5): 703-706

<http://www.wjgnet.com/1007-9327/10/703.asp>

## INTRODUCTION

Hepatic fibrosis is a common pathological process of chronic hepatic disease, leading to the development of irreversible cirrhosis<sup>[1-3]</sup>. The incidence of hepatitis is high in China<sup>[4-8]</sup>. If treated properly at fibrosis stage, cirrhosis could be prevented<sup>[9]</sup>. However, there are no effective antifibrosis drugs to date. Chinese herbs, which are well known for their long history of proven therapy of various diseases with low cost and few side effects, have particular potentials in the treatment of hepatic fibrosis<sup>[10-18]</sup>. In addition, some studies have indicated that selenium is closely related to the inhibition of hepatic fibrosis<sup>[19,20]</sup>. In the present study, we first established a rat model of chronic liver injury - hepatic fibrosis-cirrhosis and then tested the therapeutic effects of Chinese herbs and selenium on hepatic fibrosis. An array of indexes in proteins and mRNA levels were evaluated in order to understand the mechanism underlying the effects observed.

## MATERIALS AND METHODS

### Reagents

TGF- $\beta$ <sub>1</sub> mRNA primers were purchased from Sangon Biological Technology Company, China. Anti-TGF- $\beta$ <sub>1</sub> monoclonal antibody and anti-NF- $\kappa$ B polyclonal antibody were purchased from Santa Cruz Biological Technology Company, USA. Streptomycin avidin peroxidase immunohistochemistry kit for immunohistochemistry and RNA isolation kit were purchased from Boster Biological Technology Ltd, China. Anti-CD<sub>4</sub> and CD<sub>8</sub> polyclonal antibodies were purchased from Caltag Biological Technology Company, USA. Serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by the Laboratory Department of 4th affiliated hospital, Hebei Medical University, China. Serum hyaluronic acid (HA) and laminin (LN) concentrations were measured radioimmunologically using a commercial kit (Shanghai Navy Medical Institute, China).

### Preparation of Chinese herbs

The anti-fibrosis herbs included *Salvia miltiorrhiza*, *Sparganium stoloniferum*, *Angelica sinensis*, *Amyda sinensis*, *Curcuma aromatica*, *Carex phacota*. These were purchased from Shijiazhuang Lerentang Pharmacy, China. The herbs were boiled with water and extracted by alcohol: put 95% alcohol in liquid of anti-fibrosis herbs, mixed the alcohol and herbs, filtrate the protein and amyllum, then heat the liquid at 90-95 °C to evaporate the remained alcohol.

### Establishment of animal model

Wistar rats, half males-half females, weighing 180-200 g were obtained from Experimental Animal Center of Hebei Medical

University, China. The rats were housed 5 heads per cage and subjected to 12 h-d/12 h-night cycle with free access to basic food and water. All animals were treated humanely according to the national guideline for the care of animals in the country.

Hepatic fibrosis was induced in rats by carbon tetrachloride ( $\text{CCl}_4$ ). Wister rats were randomly assigned to normal control group (10), model control group (13), Colchicine group (10), anti-fibrosis herbs group (AF group, 10) and anti-fibrosis herbs plus selenium group (AS group, 10). On the first day of experiment, the rats in model group, colchicine group, AF group and AS group were given hypodermic injection of bean oil solution containing 400g/L  $\text{CCl}_4$  (0.5 mL/100 g body mass), followed by injection of the same solution (0.3 mL/100 g body mass) every 4 d. The rats in normal control group received hypodermic injection of bean oil at the same dose and frequency. Fourteen times after  $\text{CCl}_4$  administration, 3 rats in model control group were sacrificed to evaluate the liver histological change, which indicated the development of chronic hepatitis. Then colchicine group was given colchicine orally at a dose of 0.01 mg/100 g body weight daily, AF group was given anti-fibrosis herbs (2.11 g/mL) orally at a dose of 0.5 mL/100 g body mass daily, AS group was given orally anti-fibrosis herbs containing sodium selenite ( $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$ ) at 3  $\mu\text{g/mL}$  daily. Normal control group was given saline orally at a dose of 0.5 mL/100 g body mass daily. All the administrations lasted for 6 weeks.

#### Collection of specimens

At the end of the 6<sup>th</sup> week of the administration, rats in each group were sacrificed by amobarbital sodium anesthesia. Midline laparotomy was performed. Livers and thymus were excised and blood was collected through cardiopuncture.

#### Histological grading

Liver tissues were fixed in formalin and embedded in paraffin. Hematoxylin and eosin (HE) staining and Masson staining were performed according to the standard procedure. Histological grade of chronic hepatic fibrosis was determined by a semi-quantitative method based on the criteria described below: grade 0: normal liver, grade 1: few collagen fibrils extended from the central vein and portal tract, grade 2: collagen fibrils extension was apparent but had not yet encompassed the whole lobule, grade 3: collagen fibrils extended into and encompassed the whole lobule, grade 4: diffuse extension of collagen fibrils and pseudo-lobule formed.

Two pathologists who had no knowledge of their sources and each other's assessment examined the stained slide independently.

#### Flow cytometry of $\text{CD}_4$ and $\text{CD}_8$ positive cells

Sample fluorescence staining was performed using indirect immunofluorescence labeling method. Sample cells were washed in 10 mL Na-azide-PBS and centrifuged. Primary mAb to human  $\text{CD}_4$  and  $\text{CD}_8$  was added to each tube. The tube was vortexed and incubated at 37 °C for 30 min, 10 mL azide-PBS was added for inactivation and the cells were centrifuged. The supernatant was sucked away. The second antibody of FITC-IgG was added to each tube. The tube was vortexed and incubated in the dark at 37 °C for 30 min. 10 mL azide-PBS was added for inactivation and the cells were centrifuged. The samples were stored at 4 °C in the dark for FACS analysis. The primary antibody and secondary antibody were replaced by 30g/L BSA in azide-PBS as negative controls, the primary antibody was replaced by 30g/L BSA in azide-PBS as the second antibody control. The stained samples were analyzed in a FACS 420 flow cytometer (FACS 420 Fluorescence Activated Cell Sorting, Becton. Dickinson, Sunnyvale, California, USA.) The

light source was a 2W argon ion laser using a wave-length of 488 nm. The working power was 300 mW. Single parameter was measured respectively. Usually, 10 000 cells for each sample were analyzed. The analytic data were processed with a HP-Consort 30 computer. The coefficient of variation of the instrument was adjusted within 5% using PI staining chicken red blood cells.

#### Immunohistochemistry

Liver samples were formalin-fixed, paraffin-embedded and sectioned serially at 5  $\mu\text{m}$  thickness. Immunohistochemistry was performed as described in streptomyacin avidin peroxidase immunohistochemistry kit (Boster). The sections were treated with 30mL/L  $\text{H}_2\text{O}_2$  methanol at room temperature for 10 min and then washed with PBS for 5 min. After antigen retrieval, nonspecific binding sites were blocked by normal non-immune goat serum. The sections were incubated with primary antibody overnight at 4 °C, secondary antibody at 37 °C for 30 min, and avidin peroxidase at 37 °C for 20 min, followed by DAB visualization. After several washings, the sections were counterstained with hematoxylin. Negative control slides were treated with PBS.

#### Semi-quantitative PCR

Total RNA was extracted using an RNA isolation kit, and quantity and quality of the RNA extracted were measured on a spectrophotometer. Purified RNA 2  $\mu\text{g}$  and primer Oligo (dT) were used for reverse transcription (Promega). 5  $\mu\text{L}$  reverse transcription template was used for amplification through PCR. The primers were: TGF- $\beta_1$ , 113 bp: forward: 5' -AGGGCTACCATGCCACTTC-3', reverse: 5' -GCGGCACGCAGCACGGTGAT-3', GAPDH, 299 bp: forward: 5' -GTGAAGGTCGGAGTCAACG-3', reverse: 5' -GGTGAAGACGCCAGTGGACTC-3'. Amplification conditions included initial denaturation for 5 minutes at 94 °C, 30 cycles of amplification with denaturation at 94 °C for 45 seconds, annealing at 61 °C for 45 s, and extension at 72 °C for 1 min. PCR products were analyzed by agarose gel electrophoresis (15 g/L) and visualized by ethidium bromide staining and ultraviolet illumination. Expression of each TGF- $\beta_1$  was scanned by a computer. The obtained values were related to housekeeping gene GAPDH, and the resulting relative ratios were analyzed statistically.

#### Statistical analysis

Data were analyzed with SPSS software. Quantitative data were presented as mean $\pm$ SD and compared using one way ANOVA procedure. Frequency data were compared using Ridit procedure.

## RESULTS

#### Anti-fibrosis herbs and anti-fibrosis herbs plus selenium treatment suppressed fibril deposition in and ameliorated liver function of hepatic fibrosis

Specimens from normal control group showed normal structures. Specimens from model control group showed apparent formation of fibrotic septa, encompassing regenerated hepatocytes into pseudo-lobules. Regenerated hepatocytes underwent severe lipid degeneration. Specimens from AF and AS groups showed only mild fibrogenesis without pseudo-lobule formation. Statistical analysis presented significant differences between either AF group or AS group and model control group in histological gradings, indicating that fibrogenesis in both AS and AF groups was much less severe than that in colchicine group and model control group (Table 1).

Serum content of ALT, AST, HA and LN in AF group and AS group was slightly higher than that of normal control

group, but significantly lower than that in model control group ( $P<0.05$ ). Serum content of ALT, AST, HA and LN in AF group and AS group was also lower than that in colchicine group ( $P<0.05$ ). These data confirmed the histological findings that anti-fibrosis herbs and anti-fibrosis herbs plus selenium could inhibit hepatic fibrogenesis and ameliorate liver function (Table 2).

**Table 1** Histological grading of hepatic fibrosis

Group	Grade 0	Grade I	Grade II	Grade III	Grade IV
Normal	10	0	0	0	0
Model	0	0	0	0	8 <sup>c</sup>
Colchicine	0	1	3	4	1 <sup>c</sup>
AF	4	3	1	1	0 <sup>a</sup>
AS	3	3	2	1	0 <sup>ac</sup>

<sup>a</sup> $P<0.05$ , vs colchicine group; <sup>c</sup> $P<0.05$ , vs normal control group.

**Table 2** Serum content of ALT, AST, HA and LN

Group	ALT (u/L)	AST(u/L)	HA(μg/L)	LN (μg/L)
Normal	52.5±9.2 <sup>a</sup>	137.8±18.7 <sup>a</sup>	178±58 <sup>a</sup>	59.8±21.8 <sup>a</sup>
Model	165.6±32.7 <sup>ac</sup>	257.4±22.6 <sup>c</sup>	550±68 <sup>c</sup>	130.0±30.5 <sup>c</sup>
Colchicine	96.2±20.9 <sup>c</sup>	215.8±24.6 <sup>c</sup>	416±135 <sup>c</sup>	106.3±14.2 <sup>c</sup>
AF	65.8±26.5 <sup>a</sup>	150.8±34.0 <sup>a</sup>	228±83 <sup>ac</sup>	85.9±15.0 <sup>c</sup>
AS	67.3±18.4 <sup>a</sup>	154.6±27.3 <sup>a</sup>	216±58 <sup>ac</sup>	80.6±18.6 <sup>c</sup>

<sup>a</sup> $P<0.05$ , vs colchicine group; <sup>c</sup> $P<0.05$ , vs normal control group; <sup>e</sup> $P>0.05$ , vs control group.

#### **Anti-fibrosis herbs and anti-fibrosis herbs plus selenium treatment enhanced immunity of rats with hepatic fibrosis**

The percentage of CD<sub>4</sub> and CD<sub>8</sub> and the ratio of CD<sub>4</sub> to CD<sub>8</sub> were significantly lower in model control group than that in normal control group. Both AF group and AS group showed a lower percentage of CD<sub>4</sub> and a lower ratio of CD<sub>4</sub> to CD<sub>8</sub> than normal control group. However, these values were significantly higher than those in colchicine group, suggesting that anti-fibrosis herbs and anti-fibrosis herbs plus selenium could enhance the immunity of rats with hepatic fibrosis (Table 3).

**Table 3** Content of CD<sub>4</sub> and CD<sub>8</sub> in thymus

Group	Rats	CD <sub>4</sub> (%)	CD <sub>8</sub> (%)	CD <sub>4</sub> /CD <sub>8</sub>
Normal	10	54.1±1.4 <sup>a</sup>	34.1±1.2	1.58
Model	8	40.2±2.1 <sup>c</sup>	31.7±1.3	1.26 <sup>c</sup>
Colchicine	9	42.1±2.0 <sup>c</sup>	32.1±0.9	1.31 <sup>c</sup>
AF	9	50.8±3.8 <sup>a</sup>	34.8±2.1	1.45
AS	9	52.6±3.4 <sup>a</sup>	35.9±2.2	1.46

<sup>a</sup> $P<0.05$ , vs Colchicine group; <sup>c</sup> $P<0.05$ , vs normal control group.

#### **Anti-fibrosis herbs and anti-fibrosis herbs plus selenium treatment reduced TGF-β<sub>1</sub> expression**

Positive staining of TGF-β<sub>1</sub> was found at central vein and Disse's areas but not at hepatocytes on sections of normal controls, whereas on sections of model control group, the positive staining was seen at interstitial cells, inflammatory cells, impaired hepatocytes as well as normal hepatocytes. Fibrotic septa were only slightly stained.

Compared with model control group, the staining index of TGF-β<sub>1</sub> in AF and AS groups was markedly decreased ( $P<0.05$  in both groups). TGF-β<sub>1</sub> mRNA was detected in normal rat liver, but the expression level was increased significantly in

model control group. Compared with colchicine group, TGF-β<sub>1</sub> level in AF and AS groups was markedly decreased ( $P<0.05$ , Table 4). Thus, the data at both transcript and protein levels suggested that anti-fibrosis herbs and anti-fibrosis herbs plus selenium could reduce TGF-β<sub>1</sub> expression in hepatic fibrosis.

**Table 4** Level of TGF-β<sub>1</sub> mRNA in relation to GAPDH

Group	Rats	Ratio
Normal	10	0.57±0.11
Model	8	2.34±0.43 <sup>c</sup>
Colchicine	9	1.88±0.21 <sup>c</sup>
AF	9	1.07±0.31 <sup>a</sup>
AS	9	0.98±0.21 <sup>a</sup>

<sup>a</sup> $P<0.05$ , vs Colchicine group; <sup>c</sup> $P<0.05$ , vs normal control group.

#### **Anti-fibrosis herbs and anti-fibrosis herbs plus selenium treatment reduced NF-κB expression**

Positive staining of NF-κB was not found on sections of normal control group. In model control group, NF-κB was extensively expressed in nuclei of hepatocellular cells. The cells positive for NF-κB were diffusely distributed. After treatment with anti-fibrosis herbs and anti-fibrosis herbs plus selenium, the level of NF-κB staining decreased markedly.

## **DISCUSSION**

Hepatic fibrosis is a common pathological process of chronic hepatic disease, resulting in development of irreversible cirrhosis in patients. In recent years, the mechanism of development of hepatic fibrosis has been partly disclosed<sup>[2,4,5]</sup>. If treated properly at fibrosis stage, cirrhosis could be prevented. The present study demonstrated that administration of anti-fibrosis herbs and selenium was effective in treating hepatic fibrosis in rats based on both histological examination and functional analysis. The underlying therapeutic mechanism may involve enhanced immunity and down regulation of the expression of NF-κB and TGF-β<sub>1</sub>.

There are various kinds of chronic liver injuries all over the world, causing great affliction to patients. The incidence of hepatitis in China is high. Searches for effective ways to inhibit fibrogenesis and to prevent the development of cirrhosis are of great significance. Although many agents were tested, there have been no satisfactory agents with ascertained effectiveness and few side effects. Colchicine has been commonly used for anti-fibrosis<sup>[21]</sup>, but its side effect is high and its clinical use is, therefore, limited. Chinese herbs, well known for their wide range of effectiveness and low prices and few side effects, have particular potentials in the treatment of hepatic fibrosis. In this study anti-fibrosis herb treatment for chronic liver injury in rats, prevented hepatic fibrosis from developing of cirrhosis was shown by histological grading. HA and LN have been found to be good serum markers of hepatic fibrogenesis<sup>[22]</sup>. We showed that the serum content of HA and LN in AF group and AS group dropped markedly when compared with colchicine group, indicating that anti-fibrosis herb could prevent hepatic fibrogenesis. Anti-fibrosis herb could also enhance the immunity of the body by increasing the percentage of CD<sub>4</sub> and the ratio of CD<sub>4</sub> to CD<sub>8</sub> in AF group, especially in AS group, compared with that in colchicine group.

To understand the mechanism, we evaluated the effect of anti-fibrosis herb treatment on the expression of TGFβ<sub>1</sub> at both the protein and mRNA levels as TGFβ<sub>1</sub> has been considered to be the key cytokine in acceleration of the cirrhotic procession and over expression of this cytokine was closely associated with fibrogenesis in many ways<sup>[23-27]</sup>. Our results showed that

both TGF $\beta_1$  and its mRNA expression decreased significantly in AF group and AS group compared with those in control groups, indicating that anti-fibrosis herb down-regulated the expression of this cytokine, which may have contributed to the reduction of fibrosis.

NF- $\kappa$ B is known to be a family of dimeric transcription factors. It was ubiquitously expressed in non-B cells as an inactive form sequestered in cytoplasm by binding to specific inhibitory proteins termed I- $\kappa$ B<sup>[28-32]</sup>. When cells were stimulated by inducing agents, the I- $\kappa$ B became phosphorylated, ubiquitinated, and degraded. Degradation of I- $\kappa$ B could free NF- $\kappa$ B, which was then translocated into the nucleus, where it activate transcription<sup>[33-35]</sup>. NF- $\kappa$ B/Rel has been shown to be implicated in the inflammatory response and synthesis of adhesion molecules. Furthermore, NF- $\kappa$ B has been found to be related to cell proliferation and transformation<sup>[36]</sup>. Down-regulation of NF- $\kappa$ B/Rel activity could decrease the transcription of TGF $\beta_1$  to reduce the liver injury.

## REFERENCES

- 1 **Missale G**, Ferrari C, Fiaccadori F. Cytokine mediators in acute inflammation and chronic course of viral hepatitis. *Ann Ital Med Int* 1995; **10**: 14-18
- 2 **Wang YJ**, Sun ZQ. Advance in cytology and molecular biology investigation in liver fibrosis. *Xin Xiaohua Bingxue Zazhi* 1994; **2**: 244-246
- 3 **Wang FS**, Wu ZZ. Current situation in studies of gene therapy for liver cirrhosis and liver fibrosis. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 371-373
- 4 **Zhu YH**, Hu DR, Nie QH, Liu GD, Tan ZX. Study on activation and c-fos, c-jun expression of *in vitro* cultured human hepatic stellate cells. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 299-302
- 5 **Du WD**, Zhang YE, Zhai WR, Zhou XM. Dynamic changes of type I, III and IV collagen synthesis and distribution of collagen-producing cells in carbon tetrachloride-induced rat liver fibrosis. *World J Gastroenterol* 1999; **5**: 397-403
- 6 **Huang ZG**, Zhai WR, Zhang YE, Zhang XR. Study of heteroserum-induced rat liver fibrosis model and its mechanism. *World J Gastroenterol* 1998; **4**: 206-209
- 7 **Jia JB**, Han DW, Xu RL, Gao F, Zhao LF, Zhao YC, Yan JP, Ma XH. Effect of endotoxin on fibronectin synthesis of rat primary cultured hepatocytes. *World J Gastroenterol* 1998; **4**: 329-331
- 8 **Cheng ML**, Wu YY, Huang KF, Luo TY, Ding YS, Lu YY, Liu RC, Wu J. Clinical study on the treatment of liver fibrosis due to hepatitis B by IFN- $\alpha_1$  and traditional medicine preparation. *World J Gastroenterol* 1999; **5**: 267-269
- 9 **Riley TR 3rd**, Bhatti AM. Preventive strategies in chronic liver disease: part II. Cirrhosis. *Am Fam Physician* 2001; **64**: 1735-1740
- 10 **Liu YK**, Shen W. Inhibitive effect of cordyceps sinensis on experimental hepatic fibrosis and its possible mechanism. *World J Gastroenterol* 2003; **9**: 529-533
- 11 **Ma X**, Qiu DK, Xu J, Zeng MD. Effects of Cordyceps polysaccharides in patients with chronic hepatitis C. *Huaren Xiaohua Zazhi* 1998; **6**: 582-584
- 12 **Yang Q**, Yan YC, Gao YX. Inhibitory effect of Quxianruangan Capsulae on liver fibrosis in rats and chronic hepatitis patients. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 1246-1249
- 13 **You H**, Wang B, Wang T. Proliferation and apoptosis of hepatic stellate cells and effects of compound 861 on liver fibrosis. *Zhonghua Ganzhangbing Zazhi* 2000; **8**: 78-80
- 14 **Nan JX**, Park EJ, Kim YC, Ko G, Sohn DH. Scutellaria baicalensis inhibits liver fibrosis induced by bile duct ligation or carbon tetrachloride in rats. *J Pharm Pharmacol* 2002; **54**: 555-563
- 15 **Wang QC**, Shen DL, Zhang CD, Xu LZ, Nie QH, Xie YM, Zhou YX. Effect of Rangansuopiwan in expression of tissue inhibitor of metalloproteinase-1/2 in rat liver fibrosis. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 379-382
- 16 **Shen M**, Qiu DK, Chen Y, Xiong WJ. Effects of recombinant augmenter of liver regeneration protein, danshen and oxymatrine on rat fibroblasts. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 1129-1133
- 17 **Wang XL**, Liu P, Liu CH, Liu C. Effects of coordination of FZHY decoction on functions of hepatocytes and hepatic satellite cells. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 663-665
- 18 **Yao XX**, Tang YW, Yao DM, Xiu HM. Effect of yigan decoction on the expression of type I, III collagen proteins in experimental hepatic fibrosis in rats. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 263-267
- 19 **Hang M**, Song G, Minuk GY. Effects of hepatic stimulator substance, herbal medicine, Selenium/Vitamin E, and ciproloxacin on cirrhosis in the rat. *Gastroenterology* 1996; **110**: 1150-1155
- 20 **Buljebac M**, Roimic Z, Vuclic B. Serum selenium concentration in patients with liver cirrhosis hepatocellular carcinoma. *Acta Med Crearice* 1996; **50**: 11-15
- 21 **Weng HL**, Cai WM, Liu RH. Animal experiment and clinical study of effect of gamma interferon on hepatic fibrosis. *World J Gastroenterol* 2001; **7**: 42-48
- 22 **Li BS**, Wang J, Zhen YJ, Liu JX, Wei MX, Sun SQ, Wang SQ. Experimental study on serum fibrosis markers and liver tissue pathology and hepatic fibrosis in immuno-damaged rats. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 1031-1034
- 23 **Bissell DM**. Chronic liver injury, TGF-beta, and cancer. *Exp Mol Med* 2001; **33**: 179-190
- 24 **Friedman SL**. Cytokines and fibrogenesis. *Semin Liver Dis* 1999; **19**: 129-140
- 25 **Chen WX**, Li YM, Yu CH, Cai WM, Zheng M, Chen F. Quantitative analysis of transforming growth factor beta 1 mRNA in patients with alcoholic liver disease. *World J Gastroenterol* 2002; **8**: 379-381
- 26 **Gressner AM**, Weiskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. *Front Biosci* 2002; **7**: d793-807
- 27 **Lewindon PJ**, Pereira TN, Hoskins AC, Bridle KR, Williamson RM, Shepherd RW, Ramm GA. The role of hepatic stellate cells and transforming growth factor-beta (1) in cystic fibrosis liver disease. *Am J Pathol* 2002; **160**: 1705-1715
- 28 **Beraud C**, Henzel WJ, Baeuerle PA. Involvement of regulatory and catalytic subunits of phosphoinositide 3-kinase in NF- $\kappa$ B activation. *Proc Natl Acad Sci U S A* 1999; **96**: 429-434
- 29 **Regnier CH**, Song HY, Gao X, Goeddel DV, Cao Z, Rothe M. Identification and characterization of an I $\kappa$ B kinase. *Cell* 1997; **90**: 373-383
- 30 **Zandi E**, Karin M. Bridging the gap: composition, regulation, and physiological function of the I $\kappa$ B kinase complex. *Mol Cell Biom* 1999; **19**: 4547-4551
- 31 **May MJ**, Ghosh S. I $\kappa$ B kinases: Kinsmen with different crafts. *Science* 1999; **284**: 271-273
- 32 **Baeuerle PA**. I $\kappa$ B-NF- $\kappa$ B structures: at the interface of inflammation control. *Cell* 1999; **95**: 729-731
- 33 **Baeuerle PA**, Henkel T. Function and activation of NF- $\kappa$ B in the immune system. *Annu Rev Immunol* 1994; **12**: 141-179
- 34 **Ozes ON**, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF- $\kappa$ B activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* 1999; **401**: 83-85
- 35 **Romashkova JA**, Makarov SS. NF- $\kappa$ B is a target of AKT in anti-apoptotic PDGF signalling. *Nature* 1999; **401**: 86-90
- 36 **Hinz M**, Krappmann D, Eichten A, Scheidereit C, Strauss M. NF- $\kappa$ B function in growth control: regulation of cyclin D1 expression and G0-G1-to-S-phase transition. *Mol Cell Biol* 1999; **19**: 2690-2698

Edited by Liu HX and Wang XL



• CLINICAL RESEARCH •

# Population based study of noncardiac chest pain in southern Chinese: Prevalence, psychosocial factors and health care utilization

Wai Man Wong, Kwok Fai Lam, Cecilia Cheng, Wai Mo Hui, Harry Hua-Xiang Xia, Kam Chuen Lai, Wayne H.C. Hu, Jia Qing Huang, Cindy L.K. Lam, Chi Kuen Chan, Annie O.O. Chan, Shiu Kum Lam, Benjamin Chun-Yu Wong

**Wai Man Wong, Wai Mo Hui, Harry Hua-Xiang Xia, Kam Chuen Lai, Wayne H.C. Hu, Jia Qing Huang, Cindy L.K. Lam, Chi Kuen Chan, Annie O.O. Chan, Shiu Kum Lam, Benjamin Chun-Yu Wong**, Department of Medicine, The University of Hong Kong, Hong Kong SAR, China

**Kwok Fai Lam**, Department of Statistics and Actuarial Science, The University of Hong Kong, Hong Kong, China

**Cecilia Cheng**, Division of Social Science, Hong Kong University of Science and Technology, Hong Kong SAR, China

**Supported by** the Competitive Earmarked Research Grant HKU 7487/03M of the Hong Kong Research Grant Council, the Simon K. Y. Lee Gastroenterology Fund of the University of Hong Kong and the Hong Kong Society of Gastroenterology

**Correspondence to:** Dr. Benjamin Chun-Yu Wong, Department of Medicine, University of Hong Kong, Queen Mary Hospital, Pokfulam Road, Hong Kong, China. bcywong@hku.hk

**Telephone:** +852-2855-4541 **Fax:** +852-2872-5828

**Received:** 2003-09-15 **Accepted:** 2003-10-20

## Abstract

**AIM:** Population-based assessment of noncardiac chest pain (NCCP) is lacking. The aim of this study was to evaluate the prevalence, psychosocial factors and health seeking behaviour of NCCP in southern Chinese.

**METHODS:** A total of 2 209 ethnic Hong Kong Chinese households were recruited to participate in a telephone survey to study the epidemiology of NCCP using the Rose angina questionnaire, a validated gastroesophageal reflux disease (GERD) questionnaire and the hospital anxiety-depression scale. NCCP was defined as non-exertional chest pain according to the Rose angina questionnaire and had not been diagnosed as ischaemic heart diseases by a physician.

**RESULTS:** Chest pain over the past year was present in 454 subjects (20.6%, 95% CI 19-22), while NCCP was present in 307 subjects (13.9%, 95% CI 13-15). GERD was present in 51% of subjects with NCCP and 34% had consulted a physician for chest pain. Subjects with NCCP had a significantly higher anxiety ( $P<0.001$ ) and depression score ( $P=0.007$ ), and required more days off ( $P=0.021$ ) than subjects with no chest pain. By multiple logistic regression analysis, female gender (OR 1.9, 95% CI 1.1-3.2), presence of GERD (OR 2.8, 95% CI 1.6-4.8), and social life being affected by NCCP (OR 6.9, 95% CI 3.3-15.9) were independent factors associated with health seeking behaviour in southern Chinese with NCCP.

**CONCLUSION:** NCCP is a common problem in southern Chinese and associated with anxiety and depression. Female gender, GERD and social life affected by chest pain were associated with health care utilization in subjects with NCCP.

Wong WM, Lam KF, Cheng C, Hui WM, Xia HHX, Lai KC, Hu

WHC, Huang JQ, Lam CLK, Chan CK, Chan AOO, Lam SK, Wong BCY. Population based study of noncardiac chest pain in southern Chinese: Prevalence, psychosocial factors and health care utilization. *World J Gastroenterol* 2004; 10(5): 707-712  
<http://www.wjgnet.com/1007-9327/10/707.asp>

## INTRODUCTION

Noncardiac chest pain (NCCP) is a common problem and affects 23% of the U.S. population<sup>[1]</sup>. It is a benign condition with an estimated 10 year mortality of less than 1%<sup>[2]</sup>. However, the associated morbidity, as a result of inability to work and health care utilization, is enormous<sup>[3]</sup>. Population-based data of NCCP in Asia are lacking<sup>[4,5]</sup>. Furthermore, the effects of co-existing anxiety and depression on health care utilization in subjects with NCCP are unknown in Chinese. Various studies have shown an increased psychological morbidity in patients attending specialist clinics for functional gastrointestinal diseases<sup>[6-10]</sup>. This may be due to a causative effect of psychological factors on gastrointestinal symptoms, or the psychological morbidity may be a result of the functional gastrointestinal disease. Alternatively, psychological factors may influence health-seeking behaviour, and patients with co-morbid anxiety or depression may be more likely to seek medical consultation. Thus the aims of this study were to determine the population prevalence of NCCP, the effects of co-existing anxiety and depression and the health seeking behaviour of Chinese subjects with NCCP.

## MATERIALS AND METHODS

### Data collection

The telephone interview was conducted over a period of two weeks by a professional team of trained telephone interviewers from the Social Sciences Research Centre, the University of Hong Kong in November 2002. The interviewers went through intense training on the delivery of questionnaire to ensure uniformity and the questions to be understood. Random telephone numbers were generated by computer and dialed automatically. Only numbers corresponding to ethnic Chinese households were used in the study. Office numbers, facsimile machines, and non-Chinese households were excluded. Upon identification of target households, the interviewer asked to speak to the household member with the most recent birthday. This aimed to provide randomization among different members of the household. Baseline demographic data, education, occupation and income were assessed, followed by a translated version of the Rose Angina questionnaire<sup>[11]</sup>, a validated GERD questionnaire<sup>[12]</sup>, a validated translated version of the hospital anxiety depression scale<sup>[13-15]</sup> and assessment of medical care utilization and impact of the disease on social activity as described below. Overall, 3605 ethnic Chinese households were contacted by telephone. The interview was completed in

2 209 subjects (response rate=61.3%, mean age=40.3±14, 58% female). The demographic characteristics of the study subjects were comparable to the census data of Hong Kong in 2001<sup>[16]</sup>. This study was approved by the ethics committee of the University of Hong Kong.

### Sample size

A previous study demonstrated that the prevalence of NCCP was approximately 23% in Minnesota, USA<sup>[1]</sup>. To provide a 95% confidence interval ±2% and a meaningful comparison between health care seekers and non-health care seekers, we estimated a sample size of around 2000 of successful cases.

### Questionnaire

The Rose angina questionnaire is a standardized method of measuring angina and myocardial infarction in population surveys and has been validated in different ethnic groups<sup>[17-26]</sup>. 'Definite' angina was defined as chest pain that limits exertion (walking uphill or hurry, or walking at an ordinary pace on the level), is situated over the sternum or in the left chest and left arm, and is relieved within 10 min by rest<sup>[11]</sup>. 'Possible' angina was defined as chest pain that limits exertion and other criteria for definite angina not fulfilled. NCCP was defined as non-exertional chest pain according to the Rose angina questionnaire and had not been diagnosed as ischaemic heart diseases by a physician<sup>[27]</sup>. Musculoskeletal-like chest pain was defined as chest pain that worsens on breathing, movement or the presence of chest wall tenderness. The duration and characteristics of the chest pain were recorded. In the development of the Chinese version of the questionnaire, the original instrument was translated, back translated and tested for reproducibility in a sample of thirty patients attending the gastrointestinal clinic. The intraclass correlation coefficient of the translated questionnaire was 0.91. Furthermore, we tested the Rose angina questionnaire in a pilot of 50 patients with known coronary heart diseases proven by coronary angiography and 100 healthy controls. The sensitivity and specificity determined by the pilot study was 68% and 95% for the diagnosis of ischemic heart disease respectively.

Gastrointestinal symptoms were assessed by a translated Chinese version of a validated GERD questionnaire<sup>[12]</sup>. The GERD questionnaire examined the symptoms of heartburn, acid regurgitation, dyspepsia, dysphagia, globus, odynophagia, hoarseness of voice, chronic cough, asthma and pneumonia in details. In addition, past medical history, medication use, past history of esophageal, gastric, cardiac or pulmonary disease; smoking, alcohol intake, and the intake of tea and coffee were assessed. Part of the results on GERD had been presented elsewhere<sup>[28]</sup>. The severity and frequency of chest pain and other gastrointestinal symptoms were graded on a five-point Likert scale as follows: 1 (none- no symptoms / none in the past year), 2 (mild- symptoms can be easily ignored / less than once per mo), 3 (moderate- awareness of symptoms but easily tolerated / >= once per month), 4(severe- symptoms sufficient to cause an interference with normal activities / >= once weekly) and 5(incapacitating- incapacitating symptoms with an inability to perform daily activities or require day-off / >= once daily)<sup>[29]</sup>. GERD was defined as heartburn and/or acid regurgitation over the past year, which has been shown to be specific for the diagnosis of GERD<sup>[30,31]</sup>. Patients who had used non-steroidal anti-inflammatory drugs (NSAIDs) / aspirin for at least 3 d at any dosage within 3 mo prior to the survey were considered to be NSAIDs / aspirin users<sup>[32]</sup>. Anxiety and depression were assessed by the hospital anxiety and depression scale<sup>[13]</sup>. The Chinese version of this questionnaire has previously been validated<sup>[14,15]</sup> which consists of 7 questions on anxiety and 7 questions on depression.

### Medical care utilization

Medical care utilization of subjects with NCCP was classified into categories including the use of over-the-counter medication, community based medicine, accident and emergency department and admission to hospital. Impact of disease was measured by the proportion of subjects requiring days-off work due to gastrointestinal complaints, and whether subjects reported an adverse effect of the chest pain on their normal social life (social life being affected), *i.e.* symptoms sufficient to cause an interference with normal daily and social activity.

### Statistical analysis

Univariate analysis was performed by Student's *t* test for continuous variables and by chi-square test for categorical variables to assess the risk factors associated with NCCP. Multiple logistic regression analysis with sex and age adjustment was then performed to determine the risk factors associated with NCCP. Furthermore, a multiple logistic regression model was designed to determine the factors (severity and frequency of chest pain, presence of heartburn and/or acid regurgitation over the past year, gender, age, educational level (primary school or below, secondary or matriculation and tertiary), occupation (3 levels), anxiety and depression scores and social life being affected by chest pain) associated with health seeking behaviour in NCCP. To find the best model, a backward elimination stepwise procedure was carried out in a way that the factor would be eliminated from the analysis if the corresponding *P* value was greater than 0.2, in order not to miss out too much information in view of the small sample size. A *P* value of 0.05 or less was considered statistically significant and all reported *P* values were 2 sided.

## RESULTS

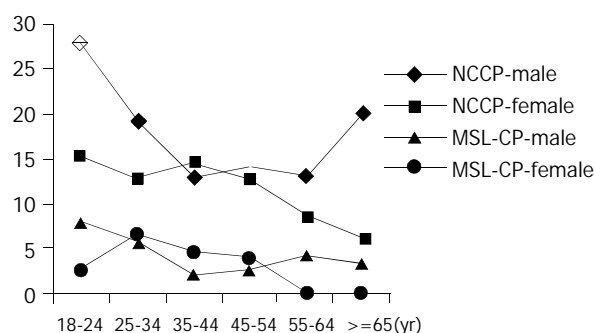
### Cardiac chest pain

Chest pain over the past year was present in 454 subjects (20.6%, 95% CI 19-22), of which 147 (6.7%) had 'possible' angina by the Rose angina questionnaire and/or ischaemic heart diseases diagnosed by a physician<sup>[11,27]</sup>.

### Noncardiac chest pain

NCCP over the past year was present in 307 subjects (13.9%, 95% CI 13-15). The demographic characteristics of subjects with NCCP, cardiac chest pain or no chest pain are given in Table 1. The prevalence of NCCP was higher in men than in women (16.6% vs 11.9%, *P*=0.002). Subjects with NCCP were significantly younger than subjects with no chest pain. However, for subjects with cardiac chest pain, the prevalence was similar between men and women (7.5% vs 8%, *P*=0.68) and the mean age was similar to subjects with no chest pain. Aspirin usage was significantly higher in subjects with cardiac chest pain than in subjects with no chest pain (20.6% vs 7.7%, *P*<0.001). NSAIDs usage was significantly higher in subjects with either NCCP or cardiac chest pain than in subjects with no chest pain. Cigarette and coffee consumption were the highest in subjects with NCCP, while alcohol consumption was similar between subjects with NCCP and subjects with cardiac chest pain. The education level and socioeconomic status were similar in subjects with NCCP, cardiac chest pain or no chest pain (Table 1). The median duration of NCCP was 24 mo (range 0.1 to 360 mo). Most (96%) subjects with NCCP had mild to moderate chest pain over the central chest area (50%) (Table 2). The frequency of chest pain was less than once per mo in three quarters of the subjects. Of the 307 subjects with NCCP, 155 (50.5%) could be classified as having GERD, while 79 (25.7%) had musculoskeletal-like chest pain. But GERD and musculoskeletal-like chest pain were overlapped

in 15.6% (48/307) of subjects. The gender difference in NCCP between men and women persisted after exclusion of subjects with musculoskeletal-like chest pain (13.1% vs 8.5%,  $P=0.001$ ). Figure 1 shows the age and sex- prevalence rates of NCCP and musculoskeletal-like chest pain over the past year. For men with NCCP over the past year, the prevalence was the highest in 18-24 age group, less common in 25-54 age group and increased again after the age of 55 (Figure 1). In contrast, the prevalence of NCCP in women was almost constant from age 18 to 44, and then dropped gradually with age. The overall prevalence of musculoskeletal-like chest pain was similar between men and women (4.2% vs 4.0%,  $P=NS$ ), but men aged 18-24 had a higher prevalence when compared to women of similar age (7.9% vs 2.6%,  $P=0.034$ ).



**Figure 1** Age and sex-specific prevalence rates (per 100) of noncardiac chest pain (NCCP) and musculoskeletal-like chest pain (MSL-CP) over the past year.

**Table 1** Demographic data of study subjects ( $n=2209$ )

	<sup>a</sup> NCCP ( $n=307$ )	<sup>a</sup> Cardiac chest pain ( $n=147$ )	<sup>a</sup> No chest pain ( $n=1755$ )
Mean age	38±14 <sup>c</sup>	42±14	41±14
Gender (M/F)	156/151 <sup>c</sup>	58/89	723/1032
Smoking (%)	20.6	16.7	16.0
Alcohol (%)	10.2 <sup>d</sup>	10.9	6.8
Aspirin user (%)	11.2	20.6 <sup>b</sup>	7.7
NSAID user (%)	11.6 <sup>c</sup>	13.9 <sup>b</sup>	6.0
Coffee (%)	22.7	14.0	18.7
Education (%)			
Primary or below	37 (13)	28 (21)	290 (18)
Secondary or matriculation	171 (61)	88 (65)	920 (57)
Tertiary	72 (26)	20 (15)	392 (25)
Total	280 (100)	136 (100)	1602 (100)
Monthly income in US\$ (%)			
None	90 (33)	60 (44)	539 (35)
1280 or below	78 (29)	34 (25)	364 (24)
1280 – 1920	39 (14)	22 (16)	254 (16)
1920 – 3200	40 (15)	10 (7)	228 (15)
>3200	25 (9)	10 (7)	160 (10)
Total	272 (100)	136 (100)	1545 (100)
Occupation (%)			
Professional and managerial	45 (16)	17 (13)	262 (16)
Technical worker and craftsmen	37 (13)	14 (10)	175 (11)
Clerical work	29 (10)	16 (12)	243 (15)
Service and sales	32 (12)	11 (8)	157 (10)
Agriculture and others	18 (7)	8 (6)	87 (6)
Non-technical worker	8 (3)	4 (3)	33 (2)
Others (students, housewife, retired and unemployed)	109 (39)	66 (49)	637 (40)
Total	278 (100)	136 (100)	1594 (100)

<sup>a</sup>The total in each individual cell may be smaller because of subject refusal, <sup>b</sup> $P<0.001$  when compared to subjects with no chest pain, <sup>c</sup> $P<0.01$  when compared to subjects with no chest pain, <sup>d</sup> $P<0.05$  when compared to subjects with no chest pain.

### Risk factors for NCCP

By univariate analysis, NCCP was associated with heartburn and/or acid regurgitation, globus, dyspepsia, feeling of acidity in stomach, alcohol and NSAID usage but not with dysphagia, odynophagia, chronic cough, hoarseness of voice, asthma, pneumonia, history of smoking, aspirin usage and coffee intake (Tables 1 and 3). By multiple logistic regression analysis, heartburn and/or acid regurgitation (OR 2.3, 95% CI 1.7-3.1), globus (OR 1.9, 95% CI 1.3-2.8), and NSAIDs use (OR 1.9, 95% CI 1.2-2.9) were independent risk factors associated with NCCP.

**Table 2** Prevalence rates and characteristics of chest pain in studied population

	Male ( $n=938$ )	Female ( $n=1271$ )	Total ( $n=2209$ )
Chest pain over the past year (%)	213 (22.7)	241 (19.0)	454 (20.6)
Possible angina by Rose angina questionnaire or ischaemic heart disease diagnosed by physicians (%)	57 (6.1)	90 (7.1)	147 (6.7)
Noncardiac chest pain-NCCP (%)	156 (16.6)	151 (11.9)	307 (13.9)
NCCP severity (%)			
Mild	69	66	67
Moderate	27	32	29
Severe or incapacitated	4	3	4
NCCP frequency (%)			
< once / month	75	76	76
≥ once / month	22	19	21
> once / weekly	3	4	4
NCCP location			
Central	46	55	50
Left	44	37	41
Right	10	8	9

**Table 3** Association of non-cardiac chest pain with other GERD symptoms over the past year

	NCCP ( $n=307$ )	No chest pain ( $n=1755$ )	OR (95% CI)
Heartburn and/or acid regurgitation (%)	50.5 <sup>a</sup>	23.2	3.4 (2.6-4.3)
Dysphagia (%)	4.1	2.8	1.5 (0.8-2.8)
Odynophagia (%)	7.1	5.8	1.2 (0.8-2.0)
Globus (%)	15.9 <sup>a</sup>	5.3	3.4 (2.3-4.9)
Dyspepsia (%)	21.8 <sup>b</sup>	14.0	1.7 (1.3-2.3)
Feeling of acidity in stomach (%)	31.3 <sup>a</sup>	16.5	2.3 (1.8-3.0)
Chronic cough (%)	8.3	5.0	1.7 (1.1-2.7)
Hoarseness of voice (%)	6.3	5.5	1.2 (0.7-1.9)
Asthma (%)	4.2	2.5	1.7 (0.9-3.3)
Pneumonia (%)	0.7	0.5	1.5 (0.3-6.9)

<sup>a</sup> $P<0.001$  when compared to subjects with no chest pain, <sup>b</sup> $P<0.01$  when compared to subjects with no chest pain.

### Health care utilization, days off work and effects of NCCP on social life

Thirty-nine percent of subjects with NCCP over the past year used one or more forms of treatment for their problems. Among the 307 subjects with NCCP over the past year, 14 (5%) purchased over the counter medication, 85 (28%) visited an outpatient clinic; 10 (3%) visited the accident and emergency department, and 11 (4%) were admitted to regional hospitals for further management. NCCP over the past year was significantly correlated with increased health care utilization ( $P<0.001$ , OR 3.2, 95% CI 2.4-4.3). A significantly higher proportion of health

seekers with NCCP had moderate to severe chest pain when compared to non-health seekers (39% vs 27%,  $P=0.025$ ) (Table 4). However, the proportion of subjects with at least monthly chest pain (28% vs 22%  $P=0.265$ ) was similar between health seekers and non-health seekers. GERD (heartburn and/or acid regurgitation) over the past year was more prevalent in NCCP health seekers than in NCCP non-health seekers (67.5% vs 40.1%,  $P<0.001$ ). A significantly higher proportion of subjects with NCCP reported their social life was affected by the chest pain (17% vs 9%,  $P<0.001$ , OR 1.9, 95% CI 1.4-2.5) and required days-off (17% vs 12%,  $P=0.021$ , OR 1.4, 95% CI 1.1-1.9) when compared to subjects with no chest pain.

### Anxiety and depression score

Subjects with NCCP symptoms over the past year had higher average anxiety (5.5 vs 4.1,  $P<0.001$ ) and depression (4.4 vs 3.8,  $P=0.007$ ) scores when compared to subjects with no chest pain. However, for subjects with NCCP symptoms, the mean anxiety score and the mean depression score were similar between NCCP subjects who had sought any medical consultation and those who had not (Table 4).

**Table 4** Comparison between subjects with noncardiac chest pain over the past year who did and did not seek health care

	NCCP health care seekers (n=131)	NCCP non-health care seekers (n=176)
Mean age $\pm$ SD	39 $\pm$ 14	36 $\pm$ 14
Female (%)	57.5 <sup>a</sup>	42.8
Chest pain severity moderate or worse (%)	39.2 <sup>a</sup>	26.5
Chest pain once a month or more (%)	27.5	21.7
Chest pain requiring days-off	35.3 <sup>b</sup>	3.1
Presence of GERD (heartburn and/or acid regurgitation) (%)	67.5 <sup>b</sup>	40.1
Social life being affected	31.4 <sup>b</sup>	6.2
Mean anxiety score	5.8	5.3
Mean depression score	4.7	4.2

<sup>a</sup> $P<0.05$  when compared to non-health seekers, <sup>b</sup> $P<0.001$  when compared to non-health seekers.

### Determinants of health care utilization in NCCP

By multiple logistic regression analysis, female gender (OR 1.9, 95% CI 1.1-3.2,  $P=0.023$ ), the presence of GERD (heartburn and/or acid regurgitation) symptoms over the past year (OR 2.8, 95% CI 1.6-4.8,  $P<0.001$ ) and social life being affected by chest pain (OR 6.9, 95% CI 3.3-15.9,  $P<0.001$ ) were independent factors associated with health seeking behaviour of subjects with NCCP.

## DISCUSSION

The epidemiology of NCCP is scanty in the literature and most studies are not population-based. Locke *et al* reported a prevalence of 23% in a semi-rural US population<sup>[1]</sup>. However, chest pain (including both exertional and non-exertional chest pain) with no past history of heart disease was the criteria for the diagnosis of NCCP in the study. Using Rose angina criteria, a population-based study in Mexican American and non-Hispanic white found a prevalence of 30% for chest pain thought not angina<sup>[33]</sup>. A UK study reported a population prevalence of 24% (Rose angina questionnaire) in 7754 subjects from 24 towns of Britain for "other chest pain"<sup>[23]</sup>. Recently, a population survey performed in Australia, using a similar definition of NCCP, reported a prevalence of 33% in

672 residents of Penrith<sup>[34]</sup>. The only independent factor for NCCP was the frequency of heartburn. No particular factor for health seeking behaviour was identified. The criteria of NCCP in our study were similar to the Australian study, but we found a considerably lower prevalence of NCCP (14%) when compared to the Western population. The exact reason is unknown but may be related to the lower prevalence of GERD (both erosive esophagitis and non-erosive reflux disease) in the Chinese population<sup>[28,32]</sup>, as GERD is the most common etiology of NCCP. The gender, age distribution and socio-economic status closely resembled those of the census data of Hong Kong in 2001, suggesting our data are highly representative<sup>[16]</sup>. Fifty-one percent of subjects with NCCP could be classified as having GERD in our study, suggesting an esophageal cause of the chest pain. Interestingly, both musculoskeletal-like chest pain and GERD were overlapped in 16% of subjects, indicating the difficulty in establishing the etiology of chest pain through a questionnaire. Furthermore, we were not able to exclude panic attacks without proper psychiatric assessment. Nevertheless, we could still obtain useful data about the impact of chest pain of presumably 'noncardiac' in origin in the Chinese community. The prevalence of NCCP was unexpectedly high in young men. It could be partially explained by the higher proportion of young men with musculoskeletal-like chest pain when compared to women of similar age. Further endoscopic and physiological studies are warranted to investigate the exact causes of chest pain in these subjects.

By multiple logistic regression analysis, the presence of heartburn and/or acid regurgitation, globus and NSAIDs intake were independent risk factors for NCCP. The symptoms of GERD and globus suggested an esophageal origin of the chest pain, but the positive association of NSAIDs intake with NCCP was interesting. It is unknown whether these subjects took NSAIDs for the symptomatic relief of chest pain or NSAID intake indirectly linked to esophageal chest pain as a result of erosive damage to the esophageal mucosa<sup>[35-38]</sup>. Furthermore, it has been shown that subjects receiving 1 500 mg aspirin per day had higher gastric mechanosensory thresholds<sup>[39]</sup>. Those who failed to increase sensory thresholds were associated with dyspepsia. Similar mechanism may operate to account for the positive association between NSAIDs and NCCP as a result of abnormal visceral perception.

In the United States, it has been estimated that US\$8 billion was spent annually for the initial care of patients who were suspected to have an acute coronary syndrome, but subsequently found not to have coronary artery disease<sup>[3]</sup>. The socio-economic effects were reflected by the higher proportion of NCCP subjects requiring days-off work and reporting an adverse effect of the illnesses on their social life when compared to subjects with no chest pain. It also concurred with our previous findings that quality of life assessment by SF-36 was significantly lower in patients with NCCP than in healthy controls<sup>[40]</sup>.

Few studies have assessed the factors associated with health care utilization in NCCP. In the Australian study mentioned above, no particular factor was found to be associated with health seeking behaviour. We found that the presence of heartburn and/or acid regurgitation were important in motivating health seeking behaviour of subjects with NCCP. Furthermore, female gender was more commonly associated with health care utilization in subjects with NCCP. Despite the higher prevalence of NCCP in men in our study, women were more likely to seek medical attention and correlated with the findings of female predominance in previous non-population based studies<sup>[41,42]</sup>. We did not find any effect of anxiety and depression on health care utilization, but subjects feeling their social life affected by chest pain were more likely

to seek help.

The major limitation of this study was the diagnosis of NCCP through a questionnaire. In clinical practice, diagnosis of NCCP requires full cardiology evaluation. However, criteria like Rome II are not available for noncardiac chest pain<sup>[43]</sup>, but rather for a sub-group of patient population only (chest pain of presumed esophageal origin). It has been shown in prospective studies that 'possible' angina or exertional chest pain was equally reliable for the prediction of future ischemic events, in both men and women<sup>[23,26]</sup>. Furthermore, self-reported history of doctor-diagnosis of angina has been shown to be a valid measure of angina in population-based studies<sup>[27]</sup>. Although the sensitivity of Rose angina questionnaire is variable, it is highly specific for the diagnosis of angina<sup>[17-26]</sup>. In our pilot study, we found that the translated Chinese version of the Rose angina questionnaire had a sensitivity and specificity of 68% and 95%. We used the combination of 'possible angina' and history of ischemic heart disease diagnosed by a physician for the diagnosis of cardiac chest pain, hoping that it would reduce the number of unrecognized ischemic heart disease in this study. Furthermore, it is impractical to perform exercise testing or coronary angiograms in all subjects with chest pain in the setting of a population-based study.

In conclusion, NCCP is a common problem in Chinese and associated with anxiety and depression. Female gender, the presence of GERD symptoms over the past year and social life affected by chest pain are independent factors associated with health care utilization in Chinese.

## ACKNOWLEDGEMENTS

The authors would like to thank the Social Science Research Centre of the University of Hong Kong for coordinating and conducting the telephone interview.

## REFERENCES

- 1 **Locke GR 3rd**, Talley NJ, Fett SL, Zinsmeister AR, Melton LJ 3rd. Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Olmsted County, Minnesota. *Gastroenterology* 1997; **112**: 1448-1456
- 2 **Chambers J**, Bass C. Chest pain with normal coronary anatomy: a review of natural history and possible etiologic factors. *Progr Cardiovasc Dis* 1990; **33**: 161-184
- 3 **Eslick GD**, Coulshed DS, Talley NJ. The burden of noncardiac chest pain. *Aliment Pharmacol Ther* 2002; **16**: 1217-1223
- 4 **Goh KL**, Chang CS, Fock KM, Ke M, Park HJ, Lam SK. Gastroesophageal reflux disease in Asia. *J Gastroenterol Hepatol* 2000; **15**: 230-238
- 5 **Wong WM**, Cheng C, Hui WM, Lam SK. Noncardiac chest pain. *Medical Progress* 2003; **30**: 15-21
- 6 **Talley NJ**, Fung LH, Gilligan IJ, McNeil D, Piper DW. Association of anxiety, neuroticism, and depression with dyspepsia of unknown cause. A case-control study. *Gastroenterology* 1986; **90**: 886-892
- 7 **Talley NJ**, Jones M, Piper DW. Psychosocial and childhood factors in essential dyspepsia. A case-control study. *Scand J Gastroenterol* 1988; **23**: 341-346
- 8 **Langeluddecke P**, Goulston K, Tennant C. Psychological factors in dyspepsia of unknown cause: a comparison with peptic ulcer disease. *J Psychosom Res* 1990; **34**: 215-222
- 9 **Harris A**, Martin BJ. Increased abdominal pain during final examinations. *Dig Dis Sci* 1994; **39**: 104-108
- 10 **Hui WM**, Shiu LP, Lam SK. The perception of life events and daily stress in nonulcer dyspepsia. *Am J Gastroenterol* 1991; **86**: 292-296
- 11 **Rose G**. The diagnosis of ischaemic heart pain and intermittent claudication in field surveys. *Bull World Health Organ* 1962; **27**: 645-658
- 12 **Locke GR**, Talley NJ, Weaver AL, Zinsmeister AR. A new questionnaire for gastroesophageal reflux disease. *Mayo Clin Proc* 1994; **69**: 539-547
- 13 **Zigmond AS**, Snaith RP. The hospital anxiety and depression scale. *Acta Psychiatr Scand* 1983; **67**: 361-370
- 14 **Leung CM**, Ho S, Kan CS, Hung CH, Chen CN. Evaluation of the Chinese version of the Hospital Anxiety and Depression Scale. A cross-cultural perspective. *Int J Psychosom* 1993; **40**: 29-34
- 15 **Lam CL**, Pan PC, Chan AW, Chan SY, Munro C. Can the Hospital Anxiety and Depression (HAD) Scale be used on Chinese elderly in general practice? *Fam Pract* 1995; **12**: 149-154
- 16 **Hong Kong Census 2001**. Census and Statistics Department, Hong Kong
- 17 **Rose GA**, Ahmeteli M, Checcacci L, Fidanza F, Glazunov I, De Haas J, Horstmann P, Kornitzer MD, Meloni C, Menotti A, van der Sande D, de Soto-Hartgrink MK, Pisa Z, Thomsen B. Ischaemic heart disease in middle-aged men. Prevalence comparisons in Europe. *Bull World Health Organ* 1968; **38**: 885-895
- 18 **Marmot MG**, Syme SL, Kagan A, Kato H, Cohen JB, Belsky J. Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California: prevalence of coronary and hypertensive heart disease and associated risk factors. *Am J Epidemiol* 1975; **102**: 514-525
- 19 **Erikssen J**, Forfang K, Storstein O. Angina pectoris in presumably healthy middle-aged men. Validation of two questionnaire methods in making the diagnosis of angina pectoris. *Eur J Cardiol* 1977; **6**: 285-298
- 20 **Krogh V**, Trevisan M, Panico S, Farinara E, Mancini M, Menotti A, Ricci G. Prevalence and correlates of angina pectoris in the Italian nine communities study. Research Group ATS-RF2 of the Italian National Research Council. *Epidemiology* 1991; **2**: 26-32
- 21 **Kutty VR**, Balakrishnan KG, Jayasree AK, Thomas J. Prevalence of coronary heart disease in the rural population of Thiruvananthapuram district, Kerala, India. *Int J Cardiol* 1993; **39**: 59-70
- 22 **Singh RB**, Sharma JP, Rastogi V, Raghuvanshi RS, Moshiri M, Verma SP, Janus ED. Prevalence of coronary artery disease and coronary risk factors in rural and urban populations of north India. *Eur Heart J* 1997; **18**: 1728-1735
- 23 **Lampe FC**, Whincup PH, Wannamethee SG, Ebrahim S, Walker M, Shaper AG. Chest pain on questionnaire and prediction of major ischaemic heart disease events in men. *Eur Heart J* 1998; **19**: 63-73
- 24 **Udol K**, Mahanonda N. Comparison of the Thai version of the Rose questionnaire for angina pectoris with the exercise treadmill test. *J Med Assoc Thai* 2000; **83**: 514-522
- 25 **Fischbacher CM**, Bhopal R, Unwin N, White M, Alberti KG. The performance of the Rose angina questionnaire in South Asian and European origin populations: a comparative study in Newcastle, UK. *Int J Epidemiol* 2001; **30**: 1009-1016
- 26 **Hart CL**, Watt GC, Davey Smith G, Gillis CR, Hawthorne VM. Pre-existing ischaemic heart disease and ischaemic heart disease mortality in women compared with men. *Int J Epidemiol* 1997; **26**: 508-515
- 27 **Lampe FC**, Walker M, Lennon LT, Whincup PH, Ebrahim S. Validity of a self-reported history of doctor-diagnosed angina. *J Clin Epidemiol* 1999; **52**: 73-81
- 28 **Wong WM**, Lai KC, Lam KF, Hui WM, Hu WHC, Lam CLK, Xia HHX, Huang JQ, Chan CK, Lam SK, Wong BCY. Prevalence, clinical spectrum and health care utilisation of gastroesophageal reflux disease in Chinese population: a population-based study. *Gastroenterology* 2003; **124**(Suppl 1): A167
- 29 **Wong WM**, Lam KF, Lai KC, Hui WM, Hu WH, Lam CL, Wong NY, Xia HHX, Huang JQ, Chan AO, Lam SK, Wong BC. Validated symptoms questionnaire (Chinese-GERDQ) for the diagnosis of gastroesophageal reflux disease in Chinese population. *Aliment Pharmacol Ther* 2003; **17**: 1407-1413
- 30 **Klauser AG**, Schindlbeck NE, Muller-Lissner SA. Symptoms in gastroesophageal reflux disease. *Lancet* 1990; **335**: 205-208
- 31 **Dent J**, Brun J, Fendrick AM, Fennerty MB, Janssens J, Kahrilas PJ, Lauritsen K, Reynolds JC, Shaw M, Talley NJ. An evidence-based appraisal of reflux disease management-the Genval Workshop Report. *Gut* 1999; **44**(Suppl 2): S1-S16
- 32 **Wong WM**, Lam SK, Hui WM, Lai KC, Chan CK, Hu WH, Xia HHX, Hui CK, Yuen MF, Chan AO, Wong BC. Long-term pro-

- spective follow-up of endoscopic oesophagitis in southern Chinese – prevalence and spectrum of the disease. *Aliment Pharmacol Ther* 2002; **16**: 2037-2042
- 33 **Mitchell BD**, Hazuda HP, Haffner SM, Patterson JK, Stern MP. High prevalence of angina pectoris in Mexican-American men. A population with reduced risk of myocardial infarction. *Ann Epidemiol* 1991; **1**: 415-426
- 34 **Eslick GD**, Jones MP, Talley NJ. Non-cardiac chest pain: prevalence, risk factors, impact and consulting - a population-based study. *Aliment Pharmacol Ther* 2003; **17**: 1115-1124
- 35 **Wilkins WE**, Ridley MG, Pozniak AL. Benign stricture of the esophagus: role of non-steroidal anti-inflammatory drugs. *Gut* 1984; **25**: 478-480
- 36 **Semble EL**, Wu WC, Castell DO. Nonsteroidal antiinflammatory drugs and esophageal injury. *Semin Arthritis Rheum* 1989; **19**: 99-109
- 37 **El-Serag HB**, Sonnenberg A. Association of esophagitis and esophageal strictures with diseases treated with nonsteroidal anti-inflammatory drugs. *Am J Gastroenterol* 1997; **92**: 52-56
- 38 **Avidan B**, Sonnenberg A, Schnell TG, Sontag SJ. Risk factors for erosive reflux oesophagitis: a case-control study. *Am J Gastroenterol* 2001; **96**: 41-46
- 39 **Holtmann G**, Gschossmann J, Buenger L, Gerken G, Talley NJ. Do changes in visceral sensory function determine the development of dyspepsia during treatment with aspirin? *Gastroenterology* 2002; **123**: 1451-1458
- 40 **Wong WM**, Lai KC, Lau CP, Hu WH, Chen WH, Wong BC, Hui WM, Wong YH, Xia HHX, Lam SK. Upper gastrointestinal evaluation of Chinese patients with non-cardiac chest pain. *Aliment Pharmacol Ther* 2002; **16**: 465-471
- 41 **Billing E**, Hjerdahl P, Rehnqvist N. Psychosocial variables in female vs male patients with stable angina pectoris and matched healthy controls. *Eur Heart J* 1997; **18**: 911-918
- 42 **Kirchgatterer A**, Weber T, Auer J, Wimmer L, Mayr H, Maurer E, Eber B. Analysis of referral diagnoses of patients with normal coronary angiogram. *Wien Klin Wochenschr* 1999; **111**: 434-438
- 43 **Clouse RE**, Richter JE, Heading RC, Janssens J, Wilson JA. Functional esophageal disorders. *Gut* 1999; **45**(Suppl 2): II31-36

Edited by Wang XL Proofread by Zhu LH



• CLINICAL RESEARCH •

# An extended assessment of bowel habits in a general population

Gabrio Bassotti, Massimo Bellini, Filippo Pucciani, Renato Bocchini, Antonio Bove, Pietro Alduini, Edda Battaglia, Paolo Bruzzi, Italian Constipation Study Group

**Gabrio Bassotti**, Sezione di Gastroenterologia ed Epatologia, Dipartimento di Medicina Clinica e Sperimentale, Università di Perugia  
**Massimo Bellini, Pietro Alduini**, Sezione di Gastroenterologia, Dipartimento di Medicina Interna, Università di Pisa  
**Filippo Pucciani**, Clinica Chirurgica Generale e Discipline Chirurgiche, Università di Firenze  
**Renato Bocchini**, UO di Medicina Polispecialistica, Azienda Sanitaria Locale, Cesena  
**Antonio Bove**, UO di Gastroenterologia ed Endoscopia Digestiva, Azienda Ospedaliera "A. Cardarelli", Napoli  
**Edda Battaglia**, Dipartimento di Fisiopatologia Clinica, Università di Torino  
**Paolo Bruzzi**, Struttura Complessa di Epidemiologia Clinica, IST Genova, Italy  
Italian Constipation Study Group (the list of all participating members of the Italian Constipation Study Group is given at the end of the paper)  
**Correspondence to:** Dr. Gabrio Bassotti, Strada del Cimitero, 2/a, 06131 San Marco (Perugia), Italy. gabassot@tin.it  
**Telephone:** +39-75-5847570  
**Received:** 2003-09-15 **Accepted:** 2003-11-06

## Abstract

**AIM:** Bowel habits are difficult to study, and most data on defecatory behaviour in the general population have been obtained on the basis of recalled interview. The objective assessment of this physiological function and its pathological aspects continues to pose a difficult challenge. The aim of this prospective study was to objectively assess the bowel habits and related aspects in a large sample drawn from the general population.

**METHODS:** Over a two-month period 488 subjects were prospectively recruited from the general population and asked to compile a daily diary on their bowel habits and associated signs and symptoms (the latter according to Rome II criteria). A total of 298 (61%) participants returned a correctly compiled record, so that data for more than 8 000 patient-days were available for statistical analysis.

**RESULTS:** The average defecatory frequency was once per day (range of 0.25-3.25) and was similar between males and females. However, higher frequencies of straining at stool ( $P=0.001$ ), a feeling of incomplete emptying and/or difficult evacuation ( $P=0.0001$ ), and manual manoeuvres to facilitate defecation ( $P=0.046$ ) were reported by females as compared to males.

**CONCLUSION:** This study represents one of the first attempts to objectively and prospectively assess bowel habits in a sample of the general population over a relatively long period of time. The variables we analyzed are coherent with the criteria commonly used for the clinical assessment of functional constipation, and can provide a useful adjunct for a better evaluation of constipated patients.

Bassotti G, Bellini M, Pucciani F, Bocchini R, Bove A, Alduini P, Battaglia E, Bruzzi P, Italian Constipation Study Group. An extended assessment of bowel habits in a general population. *World J Gastroenterol* 2004; 10(5): 713-716  
<http://www.wjgnet.com/1007-9327/10/713.asp>

## INTRODUCTION

Bowel habits are a difficult function to study objectively because of their highly private nature and negative associations. Therefore, it is not surprising that they represent one of the least understood aspects of human behaviour<sup>[1]</sup>. In the past, most knowledge of bowel habits was drawn from limited data on small groups of subjects (nurses, jail prisoners, elderly people, students)<sup>[2-5]</sup>. More recently, studies aimed generically at investigating functional gastrointestinal disorders<sup>[6-10]</sup> have yielded data on large numbers of subjects by means of telephone interviews or mailed questionnaires<sup>[11-14]</sup>. However, these studies and other reports have all been retrospective in nature and based on the subjects' assertions regarding their recent bowel function<sup>[11,15,16]</sup>. More objective investigations have assessed small groups of subjects for limited periods of time (e.g. one week)<sup>[17]</sup>.

Prospective studies on bowel habits conducted over an adequate period of time in the general population are still lacking. The aim of our prospective study was to objectively assess the frequency and characteristics of defecation in a sample of the general population over a longer period of time.

## MATERIALS AND METHODS

During a two-month period a questionnaire was consecutively distributed to 488 relatives or friends of patients attending the outpatient gastrointestinal clinic in six centres located in different regions of Italy (two in the north, two in the centre, and two in the south). A total of 259 women and 229 men received the form. To obtain the most objective possible data on bowel habits, the questionnaire took the form of a diary covering a period of 4 wk in which "yes-no" responses were to be given daily to six questions (Table 1). Drawing upon the Rome II criteria for functional constipation<sup>[18]</sup>, data on the following symptoms and signs were recorded each day, namely number of bowel movements, straining during bowel movements, feeling of incomplete emptying and/or difficult evacuation, manual manoeuvres to facilitate defecation, lumpy or hard stools. In addition, the use of laxatives was recorded.

The questionnaires were anonymous, and the only personal information the participants were required to give was their age and sex. All subjects received an exhaustive explanation about the aim of the study and the structure of the questionnaire.

Each centre received approval from the local ethics committee, the written consent of all subjects was obtained after they had been given a complete explanation of the aims of the study and the nature of the questionnaire, and the study was conducted in accordance with the Helsinki Declaration (Edinburgh revision, 2000).

For each subject, an overall score for each variable was computed as follows. The average number of bowel movements per day was obtained by taking the total number of defecations reported by the participants and divided by the total number of days in the study period (i.e., 28). The frequency in the use of laxatives was computed in the same way. The frequency of the four variables associated with defecation (straining, feeling of incomplete evacuation, need of manual help, lumpy/hard stools) was evaluated as the ratio between the total number of episodes recorded by the individual and the total number of bowel movements during the study period.

**Table 1** Four-week daily diary (Patients giving yes-no responses and number of bowel movements/day were recorded)

Questions	Time						
FIRST WEEK	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Bowel movements (number/day)							
Straining at defecation							
Feeling of incomplete defecation and/or difficult evacuation							
Manual manoeuvres							
Lumpy or hard stools							
Use of laxatives							
SECOND WEEK	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Bowel movements (number/day)							
Straining at defecation							
Feeling of incomplete defecation and/or difficult evacuation							
Manual manoeuvres							
Lumpy or hard stools							
Use of laxatives							
THIRD WEEK	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Bowel movements (number/day)							
Straining at defecation							
Feeling of incomplete defecation and/or difficult evacuation							
Manual manoeuvres							
Lumpy or hard stools							
Use of laxatives							
FOURTH WEEK	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Bowel movements (number/day)							
Straining at defecation							
Feeling of incomplete defecation and/or difficult evacuation							
Manual manoeuvres							
Lumpy or hard stools							
Use of laxatives							

### Statistical evaluation

All group means and standard deviations (SD) were calculated by averaging the individual scores. Comparisons among groups were carried out using the chi-square test or non-parametric tests. Correlations between pairs of variables were assessed by means of the non-parametric Spearman's correlation coefficient. *P* values <0.05 were chosen for rejection of the null hypothesis. Data are presented as mean±SD.

### RESULTS

A total of 298 adult subjects (163 women (54.7%), mean age 42.5±15.5 yr and 135 men (45.3%), mean age 42.4±15.9 yr) returned the completed questionnaire. The mean response rate was 61.1 % (females 62.9 %; males 58.9 %, n.s.). Therefore, data for 8 344 d were available for statistical analysis.

The distribution of the participating subjects by age and sex is shown in Table 2. Table 3 reports the frequency of defecation, expressed as the average number of evacuations per day, the frequency of pathological features and sensations at defecation, and the use of laxatives per day. Overall, the frequency of bowel movements averaged one per day (range 0.25-3.25), and was similar between males and females. No significant intra-personal variation in the parameters under examination was detected over the four-week period. Higher frequencies of straining at stool (*P*=0.001), feeling of incomplete emptying and/or difficult evacuation (*P*=0.0001),

and manual manoeuvres to facilitate defecation (*P*=0.046) were reported by females as compared to males.

**Table 2** Distribution for age and sex of population under investigation

years	Women	Men	Total
<20	3 (1.8)	3 (2.2)	6 (2)
21-30	32 (19.6)	39 (28.9)	71 (23.8)
31-40	59 (36.2)	32 (23.7)	91 (30.5)
41-50	22 (13.5)	21 (15.6)	43 (14.4)
51-60	21 (12.9)	20 (14.8)	41 (13.8)
61-70	19 (11.7)	13 (9.6)	32 (10.7)
>70	7 (4.3)	7 (5.2)	14 (4.7)

Table 4 shows the correlations between pairs of defecatory variables. Bowel movement frequency was negatively correlated with other features of defecation and the use of laxatives. Straining, a sensation of incomplete/difficult evacuation, manual manoeuvres, lumpy/hard stools and the use of laxatives were positively correlated with each other.

Concerning the relative weights of the single variables, it might be noted that 15 (5%) subjects showed a low frequency (<3/wk) of defecations, 35 (11.7%) straining during >¼ defecations, 32 (10.7%) incomplete/difficult evacuation during >¼ defecations, 2 (0.7%) manual manoeuvres during >¼ defecations, and 18 (6%) lumpy/hard stools during ¼ defecations.

**Table 3** Defecatory frequency and defecation-related variables in our population sample (data are expressed as mean±SD)

Sex	No. of defecations/day	Straining at stool/defecation	Feeling of incomplete emptying/defecation	Manual help for evacuation/defecation	Lumpy/hard stools/defecation	Use of laxatives/day
Total	1.00±0.4	0.06±0.1	0.06±0.15	0.0090±0.07	0.07±0.2	0.07±0.2
Men	1.03±0.34	0.05±0.13	0.03±0.09	0.0008±0.001	0.06±0.2	0.02±0.1
Women	0.97±0.4	0.12±0.21	0.09±0.2	0.0170±0.09	0.08±0.2	0.09±0.2
<i>P</i> (between sexes)	n.s.	0.001	0.0001	0.046	n.s.	0.001

**Table 4** Correlations among bowel habits

	Bowel mov/day	Straining	Incomplete/difficult evacuation	Manual manoeuvres	Lumpy/hard stools	Laxatives
Bowel movements (per day)	1	-0.365 <sup>a</sup>	-0.246 <sup>b</sup>	-0.123 <sup>b</sup>	-0.218 <sup>b</sup>	-0.356 <sup>b</sup>
Straining		1	0.562 <sup>b</sup>	0.293 <sup>b</sup>	0.592 <sup>b</sup>	0.416 <sup>b</sup>
Incomplete/difficult evacuation			1	0.327 <sup>b</sup>	0.558 <sup>b</sup>	0.273 <sup>b</sup>
Manual manoeuvres				1	0.303 <sup>b</sup>	0.233 <sup>b</sup>
Lumpy/hard stools					1	0.300 <sup>b</sup>
Laxatives						1

<sup>b</sup>*P*<0.01.

## DISCUSSION

Most studies on bowel habits have been based on phone interviews and on the assumption that people would report accurately, but there has been good evidence that bowel movement frequency might be misreported<sup>[19,20]</sup>. Indeed, it is very difficult to remember and report accurately one's bowel habits over recent months in a 20 min interview. Studies have shown marked discrepancies between recalled data and data that was recorded daily<sup>[19,21,22]</sup>. Moreover, people without a telephone or who were not at home when contact was attempted would be excluded from any given survey<sup>[23]</sup>. Another source of bias was the possibility that symptomatic individuals would be more keen to complete the survey process than asymptomatic subjects, which might lead to an overestimation of the frequency of symptoms.

Validated and universally accepted criteria are definitely needed if functional bowel disorders are to become a formally recognized disease entity by physicians, patients, and society<sup>[24]</sup>.

In order to circumvent some of the methodological biases discussed above, for this study a questionnaire designed to elicit the most objective possible data on individual bowel habits was drawn up. With this instrument bowel movement frequency, and sensations and characteristics related to each bowel movement were prospectively recorded by nearly 300 subjects on a daily basis for 4 wk. Moreover, to obtain a geographically representative sample of our population, participants were recruited from different parts of the country.

It may be stressed that the 61% response rate could be considered relatively high, given the nature of the data being sought. Studies employing telephone interviews or mailed questionnaires have yielded a response rate ranging from 19% to 80%. We believe that our high response rate can be attributed to the simplicity of the questionnaire (which examined only six items) and its complete anonymity. A daily dial-in service might have been more reliable, but this is costly to implement and potentially dependent on the socio-cultural environment in which the study is conducted.

It must be pointed out that due to the recruiting procedure used, the individuals who participated in this study were not selected with respect to factors such as social status, education, occupation and, possibly, the prevalence and type of bowel habits reported. However, they were prospectively recruited and not selected based on the basis of factors such as the presence/absence of pathological symptoms. Nevertheless, in light of the high participation rate, it seems reasonable to

postulate that the results of this study provide an acceptable approximation of the prevalence and type of symptoms in a general sample of Italian adults.

We are certain of our findings to be underlined. Firstly, there was a relatively large variation in bowel movement frequency, with an average of one per day, but a range of one evacuation every 4 d to about 3 bowel movements per day, with no differences in distribution between the sexes. It might also be noted that 5% of the participants reported less than 3 evacuations/week. Secondly, the number of subjects who reported abnormal features during >1/4 defecations was low, in particular, the incidence of manual manoeuvres to facilitate defecation was almost nil (0.7%). However, these variables showed a positive correlation with one another. Thirdly, the prevalence of defecation-related variables (except for the presence of lumpy/hard stools) was significantly different between the sexes, with a higher frequency in women, and interestingly, all of these are variables related with pelvic floor function. The use of laxatives was also rare (5% of the population sample), but much (*P*=0.0001) more frequent among women.

The variables analysed in this study could be helpful in the clinical assessment of functional constipation. Our data furthermore suggest that different symptoms and signs should be attributed to different weights in the evaluation of constipation scores<sup>[25,26]</sup>. For instance, a value of less than one defecation per week or the use of manual manoeuvres to facilitate defecation could represent clinically important indications for the diagnosis of constipation.

In conclusion, this study represents one of the first attempts to prospectively assess bowel habits in a general population sample over a long period of time. Further studies in "normal" subjects will obviously be needed to confirm these observations.

The following researchers of the Italian Constipation Study (ICS) Group participated in the study: Bassotti G, Chistolini F, Morelli A (Perugia); Bellini M, Alduini P, Mammini C, Rappelli L, Costa F, Stasi C, Mumolo MG, Berni I, Giorgetti S, Marchi S (Pisa); Pucciani F, Iozzi L, Cianchi F, Cortesini C (Firenze); Bocchini R, Cimatti M, Fornasari L, Montaletti I, Pazzi P (Cesena/Forlì); Bove A, Balzano A (Napoli); Battaglia E, Dughera L, Emanuelli G (Torino); Bruzzi P (Genova).

## REFERENCES

- 1 **Heaton KW**, Radvan J, Cripps H, Mountford RA, Braddon FE, Hughes AO. Defecation frequency and timing, and stool form in the general population: a prospective study. *Gut* 1992; **33**: 818-824

- 2 **Hardy TL.** Order and disorder in the large intestine. *Lancet* 1945; **i**: 519-524
- 3 **Rendtorff RC,** Kashgarian M. Stool patterns of healthy adult males. *Dis Colon Rectum* 1967; **10**: 222-228
- 4 **Milne JS,** Williamson J. Bowel habit in older people. *Gerontol Clin* 1972; **14**: 56-60
- 5 **Sandler RS,** Drossman DA. Bowel habits in apparently healthy young adults not seeking health care. *Dig Dis Sci* 1987; **32**: 841-845
- 6 **Everhart JE,** Go VLW, Johannes RS, Fitzsimmons SC, Roth HP, White LR. A longitudinal survey of self-reported bowel habits in the United States. *Dig Dis Sci* 1989; **34**: 1153-1162
- 7 **Drossman DA,** Li Z, Andruzzi E, Temple RD, Talley NJ, Thompson WG, Whitehead WE, Janssens J, Funch-Jensen P, Corazziari EUS. Householder survey of functional gastrointestinal disorders. Prevalence, sociodemography and health impact. *Dig Dis Sci* 1993; **38**: 1569-1580
- 8 **Talley NJ,** Weaver AL, Zinsmeister AR, Melton LJ. Functional constipation, and outlet delay. A population-based study. *Gastroenterology* 1993; **105**: 781-790
- 9 **Stewart WF,** Liberman JN, Sandler RS, Woods MS, Stemhagen A, Chee E, Lipton RB, Farup CE. Epidemiology of constipation (EPOC) study in the United States: relation of clinical subtypes to sociodemographic features. *Am J Gastroenterol* 1999; **94**: 3530-3540
- 10 **Pare P,** Ferrazzi S, Thompson WG, Irvine EJ, Rance L. An epidemiological survey of constipation in Canada: definitions, rates, demographics, and predictors of health care seeking. *Am J Gastroenterol* 2001; **96**: 3130-3137
- 11 **Chen LY,** Ho KY, Phua KH. Normal bowel habits and prevalence of functional bowel disorders in Singaporean adults-findings from a community based study in Bishan. *Singapore Med J* 2000; **41**: 255-258
- 12 **Boekema PJ,** van Dam van Isselt EF, Bots ML, Smout AJ. Functional bowel symptoms in a general Dutch population and associations with common stimulants. *Neth J Med* 2001; **59**: 23-30
- 13 **Icks A,** Haastert B, Enck P, Rathmann W, Giani G. Prevalence of functional bowel disorders and related health care seeking: a population-based study. *Z Gastroenterol* 2002; **40**: 177-183
- 14 **Walter S,** Hallbook O, Gotthard R, Bengmark M, Sjodahl R. A population-based study on bowel habits in a Swedish community: prevalence of faecal incontinence and constipation. *Scand J Gastroenterol* 2002; **37**: 911-916
- 15 **Levy N,** Stermer E, Steiner Z, Epstein L, Tamir A. Bowel habits in Israel. A cohort study. *J Clin Gastroenterol* 1993; **16**: 295-299
- 16 **Olubuyide IO,** Olawuyi F, Fasanmade AA. Frequency of defaecation and stool consistency in Nigerian students. *J Trop Med Hyg* 1995; **98**: 228-232
- 17 **Aichbichler BW,** Wenzl HH, Santa Ana CA, Porter JL, Schiller LR, Fordtran JS. A comparison of stool characteristics from normal and constipated people. *Dig Dis Sci* 1998; **43**: 2353-2362
- 18 **Thompson WG,** Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Müller-Lissner SA. Functional bowel disorders and functional abdominal pain. *Gut* 1999; **45** (Suppl 2): II43-II47
- 19 **Drossman DA,** Sandler RS, McKee DC, Lovitz AJ. Bowel patterns among subjects not seeking health care. Use of a questionnaire to identify a population with bowel dysfunction. *Gastroenterology* 1982; **83**: 529-534
- 20 **Manning AP,** Wyman JB, Heaton KW. How trustworthy are bowel histories? Comparison of recalled and recorded information. *BMJ* 1976; **3**: 213-214
- 21 **Whitehead WE,** Drinkwater D, Cheskin LJ, Heller BR, Schuster MM. Constipation in the elderly living at home. Definition, prevalence and relationship to lifestyle and health status. *J Am Geriatr Soc* 1989; **37**: 423-429
- 22 **Ashraf W,** Park F, Lof J, Quigley EM. An examination of the reliability of reported stool frequency in the diagnosis of idiopathic constipation. *Am J Gastroenterol* 1996; **91**: 26-32
- 23 **Thompson WG,** Irvine EJ, Pare P, Ferrazzi S, Rance L. Functional gastrointestinal disorders in Canada. First population-based survey using Rome II criteria with suggestions for improving the questionnaire. *Dig Dis Sci* 2002; **47**: 225-235
- 24 **Drossman DA.** The Rome criteria process: diagnosis and legitimation of irritable bowel syndrome. *Am J Gastroenterol* 1999; **94**: 2803-2807
- 25 **Thompson WG.** And the Working Team for functional bowel disorders. Functional bowel disorders and functional abdominal pain. In Drossman DA, Richter JE, Talley NJ, Thompson WG, Corazziari E, Whitehead WE, eds. The functional gastrointestinal disorders. Diagnosis, pathophysiology, and treatment. *Little Brown and Company Boston* 1994: 115-173
- 26 **Whitehead WE,** Bassotti G, Palsson O, Taub E, Cook EC, Drossman DA. Factor analysis of bowel symptoms in U.S. and Italian populations. *Dig Liver Dis* 2003; **35**: 774-783

Edited by Wang XL Proofread by Zhu LH

• CLINICAL RESEARCH •

# Association of *FAS* (*TNFRSF6*)-670 gene polymorphism with villous atrophy in coeliac disease

Jing Wu, BZ Alizadeh, TV Veen, JWR Meijer, CJJ Mulder, AS Peña

**Jing Wu, BZ Alizadeh, TV Veen, AS Peña**, Laboratory of Immunogenetics and Department of Gastroenterology, VU University Medical Centre, Amsterdam, PO Box 7057, 1007 MB Amsterdam, The Netherlands

**JWR Meijer, CJJ Mulder**, Departments of Pathology and Hepatogastroenterology, Rijnstate Hospital, Arnhem, The Netherlands  
**CJJ Mulder**, Head of Department of Gastroenterology, VU University Medical Center, P.O. Box 7057, 1007 MB Amsterdam, Netherlands

**Jing Wu**, Department of Gastroenterology, Jiangsu Provincial Hospital of Traditional Chinese Medicine, 210029 Nanjing, Jiangsu Province, China

**BZ Alizadeh**, Genetic Epidemiology Unit, Department of Epidemiology and Biostatistics and Department of Clinical Genetics, Erasmus Medical Centre, Rotterdam, The Netherlands

**TV Veen**, Department of Neurology, VU University Medical Centre, Amsterdam, PO Box 7057, 1007 MB Amsterdam, The Netherlands

**Supported by** the Chinese Scholarship Council, No. 98932034

**Correspondence to:** Professor A.S. Peña, MD, PhD, FRCP, VU University Medical Centre, Department of Gastroenterology, Laboratory of Immunogenetics, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands. as.pena@vumc.nl

**Telephone:** +31-20-4448416 **Fax:** +31-20-4448418

**Received:** 2003-07-17 **Accepted:** 2003-10-07

## Abstract

**AIM:** To investigate the association of *FAS* gene polymorphism with coeliac disease (CD) development.

**METHODS:** *FAS-G670A* gene polymorphism, located in a gamma interferon activation site, was studied in 146 unrelated CD patients and 203 healthy ethnically matched controls. The restriction fragment length polymorphism (RFLP) method was used to identify *FAS-G670A* gene polymorphism.

**RESULTS:** No significant difference was found in genotype frequency between CD cases and controls. In controls, however, the frequency of the GG genotype was significantly higher in women (26.5%) than in men (12.8%) ( $OR=2.44$ , 95%  $CI$  1.15-5.20,  $P=0.020$ ) and it was also higher in men with CD than controls ( $OR=2.60$ , 95%  $CI$  0.96-7.05,  $P=0.061$ ). The GG genotype frequency was significantly higher in patients with most severe villous atrophy (Marsh IIIc lesions) ( $OR=3.74$ , 95%  $CI$  1.19-11.82,  $P=0.025$ ). A significantly less proportion of men suffered from Marsh IIIc lesions than women ( $OR=0.20$ , 95%  $CI$  0.06-0.68,  $P=0.01$ ). The risk of having severe villous atrophy increased with the additive effect of the G allele in women ( $P=0.027$  for trend, age and gender adjusted).

**CONCLUSION:** *FAS-G670A* gene polymorphism is associated with the severity of villous atrophy in CD. Female gender is also associated with the severity of villous atrophy.

Wu J, Alizadeh BZ, Veen TV, Meijer JWR, Mulder CJJ, Peña AS. Association of *FAS* (*TNFRSF6*)-670 gene polymorphism with villous atrophy in coeliac disease. *World J Gastroenterol* 2004; 10(5): 717-720

<http://www.wjgnet.com/1007-9327/10/717.asp>

## INTRODUCTION

The pathogenesis of coeliac disease (CD) is unclear. Although the majority of CD patients are HLA-DQ2 or DQ8 positive, only a small percentage of HLA-DQ2 and DQ8 carriers in the healthy European population develop CD<sup>[1]</sup>. Human genome screening of CD patients and their families support the polygenic inheritance of the disease<sup>[2]</sup>. Different genes are involved in the disease susceptibility or in determining clinical course and severity of the lesion<sup>[3]</sup>. Ciccocioppo *et al*<sup>[4]</sup> reported the significant correlation between the degree of villous atrophy (VA) and the level of enterocyte apoptosis. *FAS* (*TNFRSF6*) expression increased in the abnormal segment of small intestine in CD<sup>[4,5]</sup>. Therefore, the epithelial *FAS* engagement might contribute to the development of villous atrophy<sup>[4,5]</sup>. We studied the association between the *FAS-G670A* gene polymorphism with the severity of VA and the disease susceptibility in a cohort of untreated Caucasian CD patients at the time of presentation and ethnically matched healthy controls.

## MATERIALS AND METHODS

### Subjects

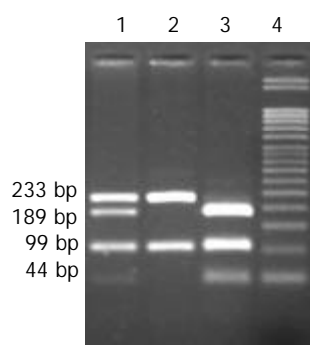
Before treatment, 146 unrelated CD patients were classified according to modified Marsh classification. Their diagnoses were confirmed only if patients responded to gluten-free diet both clinically and histologically<sup>[6]</sup>, and 203 healthy ethnically matched controls were enrolled in this study.

### Methods

**Villous atrophy classification** The histological features were classified according to the modified Marsh classification<sup>[7]</sup>. In the original classification, Marsh described subtotal villous atrophy as a destructive lesion and called it the Marsh III lesion. In our study, to assess the severity of the histological features, we classified the Marsh III type lesion into 3 subgroups<sup>[8]</sup>. Briefly, in all subgroups, histological lesions had the significant intraepithelial lymphocytosis (>30 lymphocytes per 100 epithelial cells). The architectural changes permitted classifying the lesion into three subtypes with increasing severity. They were designated as Marsh IIIa (partial VA) when the villous-crypt ratio was less than 1/1, Marsh IIIb (subtotal VA) when there were still recognizable villi in an otherwise flat mucosa, and Marsh IIIc (total VA) when there was nearly complete absence of villi.

**Typing *FAS-G670A* polymorphism** PCR amplification and genotype analysis for *FAS-G670A* were performed according to previously published methods<sup>[9]</sup>. In brief, genomic DNA was extracted from peripheral blood using a standard proteinase K digestion and phenol/chloroform extraction method and mouthwash method<sup>[10]</sup>. The *FAS-G670A* polymorphism was typed as described previously by Huang *et al*<sup>[9]</sup> with the following minor modifications. PCR: 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 62 °C, and 1 min at 72 °C, followed by a final extension for 7 min at 72 °C. Primer sequences were 5'-CTA CCT AAG AGC TAT CTA CCG TTC-3' and 5'-GGC TGT CCA TGT TGT GGC TGC-3'. The 332 bp PCR product

was digested with *Mva*I for 5 h at 37 °C. Allele G yielded three fragments of 99 bp, 189 bp, and 44 bp, whereas allele A yielded two fragments of 99 bp and 233 bp. Digested fragments were separated on 30g/L agarose gels and visualized after ethidium bromide staining (Figure 1).



**Figure 1** 1: GA genotype, 2: AA genotype, 3: GG genotype, 4: 50 bp DNA marker distinguished alleles G (189 bp) and A (233 bp).

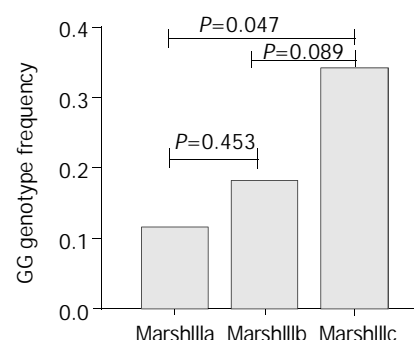
### Statistical analysis

Hardy–Weinberg equilibrium test was carried out by using statistical software for linkage analysis<sup>[11]</sup>.  $\chi^2$  statistics and Fisher's exact test were used for comparisons of frequencies. The subjects were classified according to the number of G alleles that they inherited, such as 0 for subjects who were carriers for A allele of *FAS*-G670A, *i.e.* AA genotype, 1 for those who were carriers of 1 copy of G allele, *i.e.* AG genotype and 2 for subjects who inherited 2 copies of G allele, *i.e.* GG genotype. Logistic regression was used to fit statistical models to predict the association of *FAS*-G670A polymorphism with the severity of villous atrophy or with susceptibility to CD. All the statistical models were adjusted for age (years) and gender. Associations are expressed as odd ratios (OR) with 95% confidence interval (95% CI). Estimation of 95% CI was based on Wald's method. A two tailed *P* value <0.05 was considered as significant. Statistical analysis was performed with SPSS version 10.07 for windows software.

## RESULTS

### Susceptibility of CD

The characteristics of study participants are shown in Table 1. The genotype frequencies were in Hardy–Weinberg equilibrium proportions in patients and controls. There was no significant difference in genotype distributions between patients and controls (Table 1). In men, the frequency of GG genotype was higher in cases (27.3%) compared to that of controls (12.8%), yielding to OR of 2.60, 95% CI 0.96-7.05, *P*=0.061. The GG genotype was significantly (*P*=0.020) higher in women (26.5%) compared with men (12.8%) in healthy control.



**Figure 2** Trend for GG genotype frequency in different severity of villous atrophy in untreated CD women, *P*=0.027, adjusted for age.

### Association of villous atrophy

In cases, men had a significantly lower frequency of Marsh IIIc compared to women (OR=0.20, 95% CI 0.06-0.68, *P*=0.01). Genotype and allele frequencies in Marsh III subgroups are presented in Table 2. There was a significant (*P*=0.025) difference of GG genotype frequency between Marsh IIIc which had the most severe form of the disease and Marsh IIIa (OR=3.74, 95% CI 1.19-11.82). In chromosomal analysis, we found a borderline significant association between G allele and Marsh IIIc villous atrophy (OR=1.81, 95% CI 0.97-3.38, Table 2). However, in women, G allele was significantly associated with this subgroup (OR=1.75, 95% CI 1.00-3.06, *P*=0.048).

**Table 1** Characteristics and genotype frequencies of *FAS*-G670A in untreated CD with villous atrophy

	CD (n=146)	Controls (n=203)	OR <sup>1</sup> (95%CI)	OR <sup>2</sup> (95% CI)	OR <sup>3</sup> (95% CI)
Age (mean±SD) yr.	38.7±20.0	39.1±11.9			
Gender (F/M)	113/33	117/86			
GG genotype (F/M)	25/9	31/11	1.08 (0.64-1.82)	2.44 (1.15-5.20)	0.75 (0.31-1.82)
GA genotype (F/M)	60/15	66/49	0.79 (0.51-1.22)	0.99 (0.57-1.75)	1.37 (0.63-2.99)
AA genotype(F/M)	28/9	20/26	1.30 (0.78-2.16)	0.46 (0.24-0.91)	0.88 (0.37-2.11)

F, women; M, men, OR<sup>1</sup>, age and gender adjusted, CD vs controls, OR<sup>2</sup>, age adjusted, F vs M in healthy controls, OR<sup>3</sup>, age adjusted, F vs M in CD.

**Table 2** Association of severity of villous atrophy with *FAS*-G670A polymorphism in untreated CD

Marsh	Genotype analysis			Allelic analysis			
	GG genotype F/M	GA genotype F/M	AA genotype F/M	OR <sup>1</sup> (95% CI)	G allele (%)	A allele (%)	OR <sup>2</sup> (95% CI)
IIIa (%)	3/3 (15.0)	17/6 (57.5)	6/5 (27.5)	Reference	35 (44.0)	45 (56.0)	Reference
IIIb (%)	9/5 (21.5)	25/7 (49.2)	15/4 (29.2)	1.63 (0.56-4.76)	60 (46.0)	70 (54.0)	1.10 (0.63-1.93)
IIIc (%)	13/1 (34.1) <sup>3</sup>	18/2 (48.8)	7/0 (17.1)	3.74 (1.19-11.82)	48 (58.5)	34 (41.5)	1.81 (0.97-3.38)

F, women; M, men, OR<sup>1</sup>, adjusted, for GG genotype frequency; <sup>3</sup>*P*=0.025, OR<sup>2</sup>, adjusted, for G allele frequency.



The severity of VA increased additively with increasing number of G alleles in women ( $P_{\text{for trend}}=0.027$ ) (Figure 2).

## DISCUSSION

We found a significant association between the *FAS*-670 GG genotype and the severity of celiac disease, particularly in women. However, we did not find significant differences in genotype frequencies between controls and CD patients. This suggested that the *FAS* gene was not a susceptible gene for this disease. The fact that genotype frequencies of patients and controls were in Hardy Weinberg equilibrium confirmed the validity of the typing of the results. The intestinal pathological diagnosis was confirmed by two independent pathologists. Therefore our data suggested an association of *FAS*-G670A polymorphism with the severity of CD.

Since this polymorphism of the *FAS* gene (*TNFRSF6*) is located in the promoter region, it may affect the level of transcription of the *FAS* protein. Previous work suggested that the substitution of G to A in the position -670 (TTCCAG G/A AA) would change the gamma interferon activation site (GAS) (TTCnnnGAA)<sup>[12-14]</sup>. This site was involved in interferon gamma and interferon alpha signalling<sup>[15]</sup>. GAS elements are known to bind to homodimers of a phosphorylated form of the 91-kDa transcription factor, STAT1. Interferon gamma could cause tyrosine phosphorylation of STAT1 by the interferon gamma receptor-associated Janus kinases 1 and 2. Subsequently, phosphorylated STAT1 formed homodimers and translocated into the nucleus where it induced transcription of GAS-containing genes<sup>[16]</sup>. *FAS* was significantly upregulated by interferon gamma according to several reports<sup>[17-19]</sup>. Xu *et al*<sup>[20]</sup> and De Saint Jean *et al*<sup>[21]</sup> have implicated STAT1 in this upregulation effect of interferon gamma. We therefore postulate that *FAS*-670G variant containing the GAS (TTCCAGGAA) could be affected by interferon gamma production and increase the transcription of *Fas*. This may result in different degree of apoptosis in CD with different degrees of VA. Our data confirmed that the risk of having severe villous atrophy increased additively with the number of G alleles. The GG homozygote was strongly correlated to the severity of VA in CD. Intermediated by GAS, both interferon gamma and *Fas* might play an important role in the pathogenesis of villous atrophy. This was in agreement with previous reports showing that mucosal gluten exposure elicited a high level interferon gamma expression in CD<sup>[22]</sup>. Interferon gamma could increase *FAS*-induced apoptosis of human intestinal epithelial cells in a dose-dependent manner<sup>[23]</sup>.

Considering the previous reports<sup>[24-26]</sup>, we hypothesize that *FAS*-G670A is functional and further functional tests are necessary. The *FAS*-G670A gene polymorphism contributes to the determination of the severity of small intestinal lesions and opens a new area of research that may help understand the heterogeneity of this disease. Our findings were in the same line of a recent genome-wide family-based linkage study of CD that found a potential susceptible locus at 10q23.1<sup>[27]</sup> close to the cytogenetic region (10q23.31) of the *FAS* (*TNFRSF6*) gene<sup>[28]</sup>. These results may also explain why the different genome-wide studies of families with multiple cases of CD had not uniformly found a similar lod score at chromosome 10q23, since the proportion of patients with severe villous atrophy might differ from each other in those studies.

Our data also showed a gender difference in relation to the severity of VA and in the genotype distribution of *FAS*-G670A. Even though there was no difference of GG genotype frequency in women and men among the cases, men had a significantly less proportion of Marsh IIIc VA than women. This might be due to the different immune responses between men and women. Women were more likely to develop Th1 response

(secreting higher amounts of IL-2, interferon gamma, and TNF-beta than men)<sup>[29]</sup>. In summary, our findings suggest that increased expression of *FAS* in villous atrophy is in part genetically regulated and the *FAS* gene plays a significant role.

## ACKNOWLEDGEMENTS

The authors would like to express thanks to JBA Crusius PhD, A. Zwiers MSc and Ms. M Demirkaya for their laboratory support, and Ms. L Atjak for her secretarial assistance.

## REFERENCES

- 1 **Schuppan D.** Current concepts of celiac disease pathogenesis. *Gastroenterology* 2000; **119**: 234-242
- 2 **King AL, Ciclitira PJ.** Celiac disease: strongly heritable, oligogenic, but genetically complex. *Mol Genet Metab* 2000; **71**: 70-75
- 3 **Fraser JS, Ciclitira PJ.** Pathogenesis of coeliac disease: implications for treatment. *World J Gastroenterol* 2001; **7**: 772-776
- 4 **Ciccocioppo R, Di Sabatino A, Parroni R, Muzi P, D'Alo S, Ventura T, Pistoia MA, Cifone MG, Corazza GR.** Increased enterocyte apoptosis and Fas-Fas ligand system in celiac disease. *Am J Clin Pathol* 2001; **115**: 494-503
- 5 **Maiuri L, Ciacci C, Raia V, Vacca L, Ricciardelli I, Raimondi F, Auricchio S, Quarantino S, Londei M.** Fas engagement drives apoptosis of enterocytes of coeliac patients. *Gut* 2001; **48**: 418-424
- 6 **Revised criteria for diagnosis of coeliac disease.** Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition. *Arch Dis Child* 1990; **65**: 909-911
- 7 **Marsh MN.** Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 1992; **102**: 330-354
- 8 **Rostami K, Kerckhaert J, Tiemessen R, von Blomberg BM, Meijer JW, Mulder CJ.** Sensitivity of antiendomysium and antigliadin antibodies in untreated celiac disease: disappointing in clinical practice. *Am J Gastroenterol* 1999; **94**: 888-894
- 9 **Huang QR, Morris D, Manolios N.** Identification and characterization of polymorphisms in the promoter region of the human Apo-1/Fas (CD95) gene. *Mol Immunol* 1997; **34**: 577-582
- 10 **Laine ML, Farre MA, Crusius JB, van Winkelhoff AJ, Pena AS.** The mouthwash: a non-invasive sampling method to study cytokine gene polymorphisms. *J Periodontol* 2000; **71**: 1315-1318
- 11 **Ott J.** Utility programs for analysis of genetic linkage; Program HWE; 1988; URL: <http://www.hgmp.mrc.ac.uk/Registered/Help/linkutil/>
- 12 **Decker T, Kovarik P, Meinke A.** GAS elements: a few nucleotides with a major impact on cytokine-induced gene expression. *J Interferon Cytokine Res* 1997; **17**: 121-134
- 13 **Chatterjee-Kishore M, Wright KL, Ting JP, Stark GR.** How Stat1 mediates constitutive gene expression: a complex of unphosphorylated Stat1 and IRF1 supports transcription of the LMP2 gene. *EMBO J* 2000; **19**: 4111-4122
- 14 **Bauvois B, Djavaheri-Mergny M, Rouillard D, Dumont J, Wietzerbin J.** Regulation of CD26/DPPIV gene expression by interferons and retinoic acid in tumor B cells. *Oncogene* 2000; **19**: 265-272
- 15 **Shuai K.** Interferon-activated signal transduction to the nucleus. *Curr Opin Cell Biol* 1994; **6**: 253-259
- 16 **Gao J, Morrison DC, Parmely TJ, Russell SW, Murphy WJ.** An interferon-gamma-activated site (GAS) is necessary for full expression of the mouse iNOS gene in response to interferon-gamma and lipopolysaccharide. *J Biol Chem* 1997; **272**: 1226-1230
- 17 **Schwartzberg LS, Petak I, Stewart C, Turner PK, Ashley J, Tillman DM, Douglas L, Tan M, Billups C, Mihalik R, Weir A, Tauer K, Shope S, Houghton JA.** Modulation of the Fas signaling pathway by IFN-gamma in therapy of colon cancer: phase I trial and correlative studies of IFN-gamma, 5-fluorouracil, and leucovorin. *Clin Cancer Res* 2002; **8**: 2488-2498
- 18 **Dai C, Krantz SB.** Interferon gamma induces upregulation and activation of caspases 1, 3, and 8 to produce apoptosis in human erythroid progenitor cells. *Blood* 1999; **93**: 3309-3316
- 19 **Pouly S, Becher B, Blain M, Antel JP.** Interferon-gamma modu-

- lates human oligodendrocyte susceptibility to Fas- mediated apoptosis. *J Neuropathol Exp Neurol* 2000; **59**: 280-286
- 20 **Xu X**, Fu XY, Plate J, Chong AS. IFN-gamma induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression. *Cancer Res* 1998; **58**: 2832-2837
- 21 **De Saint Jean M**, Brignole F, Feldmann G, Goguel A, Baudouin C. Interferon-gamma induces apoptosis and expression of inflammation- related proteins in Chang conjunctival cells. *Invest Ophthalmol Vis Sci* 1999; **40**: 2199-2212
- 22 **Nilsen EM**, Jahnsen FL, Lundin KE, Johansen FE, Fausa O, Sollid LM, Jahnsen J, Scott H, Brandtzaeg P. Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. *Gastroenterology* 1998; **115**: 551-563
- 23 **Ruemmele FM**, Russo P, Beaulieu J, Dionne S, Levy E, Lentze MJ, Seidman EG. Susceptibility to FAS-induced apoptosis in human nontumoral enterocytes: role of costimulatory factors. *J Cell Physiol* 1999; **181**: 45-54
- 24 **Huang QR**, Danis V, Lassere M, Edmonds J, Manolios N. Evaluation of a new Apo-1/Fas promoter polymorphism in rheumatoid arthritis and systemic lupus erythematosus patients. *Rheumatology* 1999; **38**: 645-651
- 25 **Feuk L**, Prince JA, Breen G, Emahazion T, Carothers A, St Clair D, Brookes AJ. Apolipoprotein-E dependent role for the FAS receptor in early onset Alzheimer's disease: finding of a positive association for a polymorphism in the TNFRSF6 gene. *Hum Genet* 2000; **107**: 391-396
- 26 **Bolstad AI**, Wargelius A, Nakken B, Haga HJ, Jonsson R. Fas and Fas ligand gene polymorphisms in primary Sjogren's syndrome. *J Rheumatol* 2000; **27**: 2397-2405
- 27 **King AL**, Yiannakou JY, Brett PM, Curtis D, Morris MA, Dearlove AM, Rhodes M, Rosen-Bronson S, Mathew C, Ellis HJ, Ciclitira PJ. A genome-wide family-based linkage study of coeliac disease. *Ann Hum Genet* 2000; **64**(Pt 6): 479-490
- 28 <http://genome.ucsc.edu/>, Assembly Human April 2003
- 29 **Whitacre CC**, Reingold SC, O'Looney PA. A gender gap in autoimmunity. *Science* 1999; **283**: 1277-1278

Edited by Gupat MK and Wang XL

• CLINICAL RESEARCH •

# Gallbladder contractility and volume characteristics in gallstone dyspepsia

De-Chuan Chan, Tzu-Ming Chang, Cheng-Jueng Chen, Teng-Wei Chen, Jyh-Cherng Yu, Yao-Chi Liu

**De-Chuan Chan, Cheng-Jueng Chen, Teng-Wei Chen, Jyh-Cherng Yu, Yao-Chi Liu**, Division of General Surgery, Department of Surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan, China

**Tzu-Ming Chang**, Department of Surgery, Shalu Tungs' Memorial Hospital, Tai-Chung, Taiwan, China

**Correspondence to:** Yao-Chi Liu, MD, Division of General Surgery, Department of Surgery, Tri-Service General Hospital, 325 Section 2, Cheng-Kung Road, Neihu 114, Taipei, Taiwan, China. chrischan1168@yahoo.com.tw

**Telephone:** +886-2-87927191 **Fax:** +886-2-87927372

**Received:** 2003-10-08 **Accepted:** 2003-12-30

## Abstract

**AIM:** It is difficult to differentiate gallstone dyspepsia and functional dyspepsia by clinical symptoms and signs. We hypothesized that gallstone dyspepsia was related to abnormal gallbladder motility. We aimed to differentiate gallstone dyspepsia from functional dyspepsia by measuring gallbladder motility.

**METHODS:** We measured gallbladder volume changes in response to gastric distension (saline 500 mL) and fatty meal in 10 normal volunteers (controls) and 62 patients with gallstones and dyspepsia before cholecystectomy. Forty cholecystectomized patients were symptom free or had improvement (group I), while the remaining 22 patients had persistent dyspepsia (group II). Gallbladder volume change and ejection fraction were analyzed and compared among the three groups.

**RESULTS:** In group I, there were significant decreases in gallbladder volumes 5-25 min after gastric distension, compared to fasting volumes. Compared to normal volunteers and group II, group I had significantly decreased gallbladder volumes 10-20 min after drinking 500 mL of normal saline and 10 to 50 min after eating fatty meal.

**CONCLUSION:** Our results support the hypothesis that increased gallbladder contraction after gastric distension or fatty meal may be related to dyspeptic symptoms in uncomplicated gallstone disease. These findings may be useful in differentiating functional dyspepsia from gallstone dyspepsia, patients with the latter disease may benefit from laparoscopic cholecystectomy.

Chan DC, Chang TM, Chen CJ, Chen TW, Yu JC, Liu YC. Gallbladder contractility and volume characteristics in gallstone dyspepsia. *World J Gastroenterol* 2004; 10(5): 721-724

<http://www.wjgnet.com/1007-9327/10/721.asp>

## INTRODUCTION

Laparoscopic cholecystectomy is the choice of treatment for symptomatic gallstone disease and is performed with increasing frequency. It is clear that gallstone patients with complications

(acute cholecystitis, gallstone pancreatitis or jaundice) or severe biliary pain should undergo cholecystectomy. Conversely, for asymptomatic gallstone disease, no treatment should be done. Nonetheless, some patients with uncomplicated gallstone disease, once termed gallstone dyspepsia<sup>[1]</sup>, suffer from mild abdominal symptoms, such as postprandial flatulence, bloating, nausea and belching. Most of these patients also undergo laparoscopic cholecystectomy, but about 20-30% of these cholecystectomized patients still complain of abdominal symptoms after surgery. These symptoms may be associated with preoperatively undiagnosed functional gut disease unrelated to gallstones<sup>[2-4]</sup>. In order to avoid unnecessary cholecystectomies, it is important to ascertain preoperatively that these mild symptoms of gallstone patients with dyspepsia are really caused by gallstones. New diagnostic methods to predict which patients will benefit from cholecystectomy are therefore necessary.

Gallstone dyspepsia and functional dyspepsia have coexisting symptoms and it is difficult to differentiate from each other based on the dyspeptic symptoms<sup>[5-7]</sup>. Despite numerous studies, the mechanism of gallstone dyspepsia has not been completely explained<sup>[8-10]</sup>. In the past, the majority of literature focused on the pathogenesis of gallstone formation. It is postulated that two distinct subgroups of gallstone patients can be identified with regard to gallbladder emptying, including "normal contractors" and "pathologic contractors" or "strong contractors" and "weak contractors"<sup>[11-14]</sup>. Therefore, we hypothesize that in these patients, gallstone dyspepsia may result from abnormal gallbladder motor activity stimulated by gastric distension or fatty meals. In this study, we investigated the difference in gallbladder contractility in response to gastric distension and fatty meals in healthy volunteers and patients with gallstones and dyspepsia. If so, preoperative assessment of gallbladder contractility with ultrasonography would contribute to better outcomes of cholecystectomy.

## MATERIALS AND METHODS

Gallbladder volume was assessed in 72 subjects, including 10 healthy controls and 62 gallstone patients with symptoms of dyspepsia. None of the gallstone patients had past or present signs of complicated gallstone diseases, such as acute cholecystitis, biliary pancreatitis, jaundice or severe biliary pain. Panendoscopy and abdominal ultrasound were done to exclude esophagitis, peptic ulcer, pancreatitis or other organic diseases in all gallstone patients. After an overnight fast, the fasting gallbladder volume was measured ultrasonographically three times within 5 min, with the subjects lying supine or turned partially on their sides. The gallbladder volume was estimated using a real-time ultrasound system (MK-500, ATL, Bothell, WA) with a 3.5-MHz transducer. The largest longitudinal and transverse gallbladder images were recorded. The gallbladder volume was calculated using the computerized sum-of-cylinders method as described by Hopman *et al*<sup>[15]</sup>. Then, the gallbladder volume was again measured after the patient drank 500 mL of normal saline at room temperature within a 2-min period. Measurement was made at 5 min

intervals for the first 30 min, then at 10 min intervals between 30 to 90 min after drinking. On the next day, we serially measured gallbladder volumes after the patients ate a fatty meal (fried egg cake) containing protein 9.32 g, fat 20.3 g and carbohydrate 14.4 g, 277.58 Kcal, followed by 100 mL of water. After the measurements, the gallstone patients with dyspepsia underwent laparoscopic cholecystectomy and were monitored in the outpatient department at least for 1 yr after surgery. These patients were allocated to 2 groups according to cholecystectomy outcomes. Forty patients (group I) were symptom free or had improved symptoms after cholecystectomy. Twenty-two patients (group II) complained of persistent dyspepsia after cholecystectomy, with the same symptoms as their preoperative symptoms. Thereafter, we analyzed and compared the differences among the three patient groups (groups I, II and controls) for fasting gallbladder volume, volume changes and ejection fraction in response to gastric distension and fatty meal.

The fasting gallbladder volume was calculated as the mean of three values before meal intake. The postprandial gallbladder volume was expressed as a percentage of the fasting volume. Results were expressed as mean $\pm$ SE. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software. Comparisons among the three groups were analyzed using the  $\chi^2$  test. Differences between means were considered significant at  $P<0.05$ .

## RESULTS

Patient characteristics are shown in Table 1. The fasting gallbladder volume tended to be larger in the two groups of patients with gallstones than in healthy volunteers during either test (Table 1).

**Table 1** Characteristics of subjects ( $\chi^2$  test)

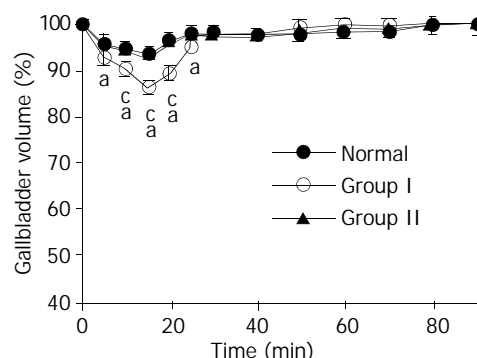
	Normal (n=10)	Group I (n=40)	Group II (n=22)	P value
Age, (yr)	61.2 $\pm$ 12.3	59.5 $\pm$ 15.1	62.5 $\pm$ 13.4	NS
Sex (M:F)	4:6	14:26	9:13	NS
Weight (kg)	60.9 $\pm$ 5.7	59.8 $\pm$ 13.8	57.6 $\pm$ 9.8	NS
BMI	27.8 $\pm$ 3.8	24.8 $\pm$ 4.7	24.4 $\pm$ 5.7	NS
Fasting gallbladder volume (mL)	26.3 $\pm$ 7.4	32.6 $\pm$ 14.1	33.9 $\pm$ 10.7	<0.05

BMI, body mass index.  $P<0.05$  was considered statistically significant.

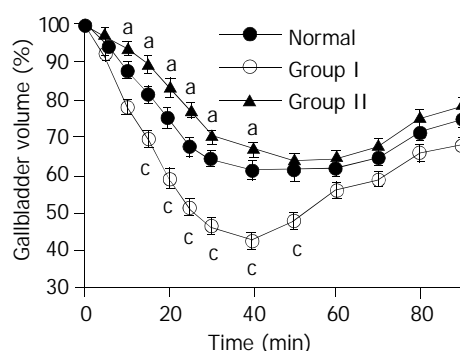
In normal volunteers and group II gallstone patients after drinking 500 mL normal saline, the gallbladder volume did not significantly change from the basal fasting level throughout the 90-min observation period (Figure 1). In contrast, in group I patients, within 5 to 25 min after drinking 500 mL of normal saline, there was a significant decrease in the gallbladder volume from the basal fasting volume ( $P<0.05$ ) (Figure 1). The gallbladder volume change (10-20 min) in group I patients was significantly larger than that in healthy volunteers and group II patients ( $P<0.05$ ) (Figure 1).

After the fatty meal, there were significant decreases in gallbladder volume in healthy volunteers and the two groups of gallstone patients throughout the 90-min study period (Figure 2). Group I patients had larger gallbladder volume changes ( $P<0.05$ ) than the other two groups during the early phase (10-50 min) of testing, the lowest residual volume was detected at the 40<sup>th</sup> min. We also found that the gallbladder volume change was significantly smaller ( $P<0.05$ ) in group II during the early phase (10-40 min) than in healthy volunteers in

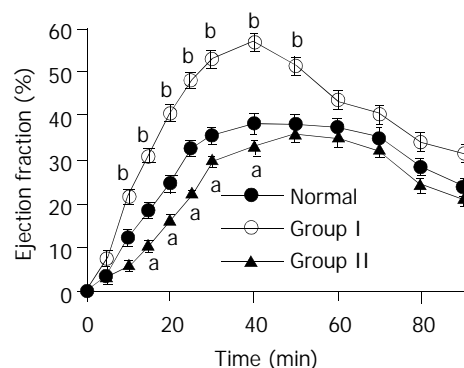
response to fatty meal (Figure 2). The ejection fraction was significantly greater ( $P<0.05$ ) in group I than the other two groups 10-50 min after the fatty meal (Figure 3).



**Figure 1** Gallbladder volume change after ingestion of 500 mL of normal saline (N.S.) in healthy volunteers (normal controls,  $n=10$ ), group I (symptom free or symptom improved after cholecystectomy) ( $n=40$ ) and group II (persistent dyspepsia after cholecystectomy) ( $n=22$ ) gallstone patients. Values are mean $\pm$ SE ( $\chi^2$  test). <sup>a</sup> $P<0.05$ : significant volume change from fasting gallbladder volume. <sup>c</sup> $P<0.05$ : significant difference among three groups (group I vs normal group and group II patients).



**Figure 2** Gallbladder volume change after fatty meal in healthy volunteers (normal,  $n=10$ ), group I (symptom free or symptom improved after cholecystectomy) ( $n=40$ ) and group II (persistent dyspepsia after cholecystectomy) ( $n=22$ ) gallstone patients. Values are mean $\pm$ SE ( $\chi^2$  test). <sup>a</sup> $P<0.05$ : significant difference between normal group and group II. <sup>c</sup> $P<0.05$ : significant differences among three groups (group I vs normals and group II patients).



**Figure 3** Ejection fraction of gallbladder in healthy volunteers and gallstone patients after oral fatty meal. Values are mean $\pm$ SE ( $\chi^2$  test). <sup>a</sup> $P<0.05$ : significant difference between group II (persistent dyspepsia after cholecystectomy) and normal group. <sup>b</sup> $P<0.05$ : significant differences among three groups (group I vs normal group and group II).

## DISCUSSION

It is clear that complicated gallstone diseases, such as acute cholecystitis, obstructive jaundice, gallstone pancreatitis and severe biliary pain, are good indications for cholecystectomy. With the technical improvements in laparoscopic surgery, many patients with uncomplicated gallstone disease termed gallstone dyspepsia underwent laparoscopic cholecystectomy for only mild gastrointestinal symptoms such as postprandial flatulence, bloating or belching. However, the benefit of cholecystectomy for gallstone patients with dyspepsia has remained debatable<sup>[16]</sup>. Only about one half of patients were symptom free after cholecystectomy<sup>[17,18]</sup>. About 20-30% of cholecystectomized patients still complained of dyspepsia, which might have been associated with preoperatively undiagnosed functional gut diseases unrelated to gallstones<sup>[2-4]</sup>. Because gallstone dyspepsia and functional dyspepsia had coexisting symptoms, it is difficult to differentiate them based on dyspeptic symptoms<sup>[5]</sup>. In order to avoid unnecessary cholecystectomies, it is important to ascertain preoperatively that the symptoms of gallstone patients with dyspepsia are truly caused by gallstones.

Even with numerous studies, the mechanisms underlying gallstone dyspepsia could not be completely explained<sup>[8-10]</sup>. Some investigators believed that dyspepsia was associated with functional gut diseases such as antroduodenal reflux and irritable bowel syndrome, rather than with gallstone disease<sup>[19,20]</sup>. However, Johnson<sup>[21]</sup> suggested that an association existed between flatulent dyspepsia and gallbladder disease, although it had no direct relationship. In the past, the majority of literature focused on the pathogenesis of gallstone formation rather than on the association of gallbladder motility and clinical symptoms. Using different techniques, investigators showed decreased<sup>[10-12]</sup>, normal<sup>[22]</sup> or even increased<sup>[23]</sup> gallbladder contractility compared to normal controls. Moreover, it has been postulated that two distinct subgroups of gallstone patients could be identified with regard to gallbladder emptying, including strong and weak contractors<sup>[24,25]</sup>. Perhaps the mild abdominal symptoms were related to abnormal gallbladder motility. We identified two distinct groups of patients with gallstones and dyspepsia in terms of gallbladder contractility.

In our study, patients with gallstones and dyspepsia had significantly larger fasting gallbladder volumes, which were similar to a previous report<sup>[26]</sup>. The symptoms experienced in gallstone patients were traditionally believed to arise from gallbladder spasm, and normal gallbladder contractility was thought to be a prerequisite for the development of symptoms. Heaton reported that gallstones were prone to cause symptoms in younger patients and cited the explanation that younger people perhaps had stronger gallbladder contractions<sup>[27]</sup>. We also found that group I patients had larger gallbladder volume changes and stronger gallbladder contractions during the early phase of observation in response to 500 mL of normal saline and fatty meals, although there was no significant difference in age among them. We found that in group I patients, symptoms improved after cholecystectomy, that is, patients who had stronger gallbladder contractions in response to gastric distension and fatty meal benefited from cholecystectomy. However, group II patients with impaired gallbladder contractility did not benefit from cholecystectomy because their symptoms were perhaps unrelated to gallbladder motility.

In 1979 Debas *et al* found that antral distension initiated a cholinergic pyloro-cholecystic reflex causing gallbladder contraction in dogs<sup>[28]</sup>. Intact vagus nerves and cholinergic pathways were required for this reflex. Vagal stimulation via mechanoreceptors in the stomach initiated gallbladder contraction independently of meal composition<sup>[29]</sup>. Yamamura *et al* found that gastric distension following ingestion of

400 mL of water induced a maximum gallbladder evacuation of 25%, compared with a maximum gallbladder contraction of 44% after ingestion of fatty meal in humans<sup>[30]</sup>. However, in our study, the gallbladder response to gastric distension following ingestion of 500 mL of normal saline in healthy volunteers and group II gallstone patients was not significantly different from fasting gallbladder volumes. In group I gallstone dyspepsia patients, after drinking 500 mL of normal saline, the lowest gallbladder residual volume (13%) occurred at 15 min, with progressive recovery to the fasting volume thereafter. This saline water induced gallbladder early net emptying most prominently between 5 and 25 min in group I patients. In contrast, after fatty meal, there were significant decreases in gallbladder volumes both in healthy volunteers and the two groups of gallstone patients throughout the study period. The group I gallstone patients had the largest gallbladder volume change at the 40<sup>th</sup> min of the study. The ejection fraction was significantly increased in group I patients compared to the other two groups 10-50 min after fatty meal, but not thereafter.

Our study showed that gallbladders of group I gallstone dyspepsia patients had greater motility than those of normal individuals and group II patients in response to both gastric distension and fatty meal, which is important for elucidating the pathophysiology of gallstone dyspepsia. To our knowledge, these findings have not been previously reported. The greater motility might have been caused by hypersensitivity of the gallbladder wall to neural stimuli and hormone or by increased serum hormone levels in patients with gallstone dyspepsia. We do not know if gallbladder hypermotility precedes the development of gallstones, accompanies their development or is a relatively late sequel of their appearance. The finding that not all gallstone patients have gallbladder hypermotility also suggests there may be mechanisms for hypermotility other than the gallstones themselves. The primary mechanism is still debated and may indeed differ among individual patients. The gallbladder hypermotility phenomenon requires further studies.

In our study, more patients (34%) did not benefit from cholecystectomy than those in other studies (20-30%). We consider that the difference in the study populations may explain this phenomenon. Previous studies included more patients with acute cholecystitis or severe biliary pain who are better candidates for cholecystectomy<sup>[2-4]</sup>. The patients in our study were selected because of their relatively mild symptoms of postprandial distress without fever, chills, transient jaundice or severe biliary pain. This is one reason that might explain why others reported better cholecystectomy results.

Most studies of gallbladder motility in the presence of gallstones have shown impaired gallbladder contractility. Thus, our findings have also been noted by others<sup>[22-25]</sup>. Our study indicated that increased gallbladder contraction was the prerequisite for the development of dyspepsia symptoms. Previous studies included more asymptomatic patients with decreased gallbladder contractions in response to gastric distension and fatty meals.

Increased gallbladder contractility in patients with gallstone dyspepsia may be useful in identifying patients with atypical mild symptoms or possibly with early acalculous cholecystitis. The studies we conducted were noninvasive, inexpensive and within the capability of conducting ultrasonography in modern hospitals. The findings of promptly increased gallbladder contractility in response to intake of 500 mL of normal saline and fatty meal may lend support to including gallbladder contractility studies as part of the dyspepsia differential diagnosis.

Postprandial gallbladder volume changes and relative ejection fraction, determined sonographically, seem to be able to discriminate gallstone dyspepsia from functional dyspepsia.

In clinical practice, this type of diagnostic study may help to determine the appropriate treatment for gallstone patients with dyspepsia.

## REFERENCES

- 1 **Barbara L**, Camilleri M, Corinaldesi R. Definition and investigation of dyspepsia. Consensus of an international ad hoc working party. *Dig Dis Sci* 1989; **34**: 1272-1276
- 2 **Ure BM**, Troidl H, Spangenberger. Long-term result after laparoscopic cholecystectomy. *Br J Surg* 1995; **82**: 267-270
- 3 **Borly L**, Anderson IB, Bardram L. Preoperative prediction model of outcome after cholecystectomy for symptomatic gallstones. *Scand J Gastroenterol* 1999; **34**: 1144-1152
- 4 **Gui GP**, Cheruvu CV, West N. Is colecystectomy effective treatment for symptomatic gallstones? Clinical outcome after long-term follow up. *Ann R Coll Surg Engl* 1998; **80**: 25-32
- 5 **Muszynski J**, Sieminska J, Zagorowicz. Comparison of clinical features of cholecystolithiasis and functional dyspepsia. *Med Sci Monit* 2000; **6**: 330-335
- 6 **Heikkinen M**, Pikkarainen P, Takala J, Rasanen H, Julkunen R. Etiology of dyspepsia: four hundred unselected consecutive patients in general practice. *Scand J Gastroenterol* 1995; **30**: 519-523
- 7 **Koch M**, Caparso G. Functional dyspepsia: how could a biliary dyspepsia sub-group be recognized? A methodological approach. *Ita J Gastroenterol* 1996; **28**: 261-268
- 8 **Crean GP**, Holden RJ, Knill-Jones RP. A database on dyspepsia. *Gut* 1994; **35**: 191-202
- 9 **Kang JY**, Yap I, Gwee KA. The pattern of functional and organic disorders in an Asian gastroenterological clinic. *J Gastroenterol Hepatol* 1994; **9**: 124-127
- 10 **Berstad A**, Hausken T, Gilja OH. Imaging studies in dyspepsia. *Eur J Surg Suppl* 1998; **582**: 42-49
- 11 **Pomeranz IS**, Shaffer EA. Abnormal gallbladder emptying in a subgroup of patients with gallstones. *Gastroenterology* 1985; **88**: 787-791
- 12 **Fan Y**, Dou YL, Dai XZ. Gallbladder hypokinesia in patients with functional dyspepsia. *Chin Nat J New Gastroenterol* 1996; **2**(Suppl 1): 114
- 13 **Thompson JC**, Fried GM, Ogden WD. Correlation between release of cholecystokinin and contraction of the gallbladder in patients with gallstones. *Ann Surg* 1982; **195**: 670-676
- 14 **Zhu XG**, Greeley GH, Newman J. Correlation of in vitro measurement of contractility of the gallbladder with *in vivo* ultrasonographic findings in patients with gallstones. *Surg Gynaecol Obstet* 1985; **161**: 470-472
- 15 **Hopman WP**, Brouwer WF, Rosenbusch G. A computerized method for rapid quantification of gall bladder volume from real-time sonograms. *Radiology* 1985; **154**: 236-237
- 16 **Paul A**, Troidl H, Gay K. Dyspepsia and food intolerance in symptomatic gallstone disease. Does cholecystectomy help? *Chirurg* 1991; **62**: 462-466
- 17 **Ros E**, Zambon D. Postcholecystectomy symptoms> a prospective study of gallstone patients before and two years after surgery. *Gut* 1987; **28**: 1500-1504
- 18 **Johnson AG**. Cholecystectomy and gallstone dyspepsia-clinical and physiological study of a symptom complex. *Ann Royal Coll Surg Engl* 1975; **56**: 69-80
- 19 **Egbert AM**. Gallstone symptoms. Myth and reality. *Postgrad Med* 1991; **90**: 119-126
- 20 **Talley NJ**. Gallstones and upper abdominal discomfort. Innocent bystander or a cause of dyspepsia? *J Clin Gastroenterol* 1995; **20**: 182-183
- 21 **Howard PJ**, Murphy GM, Dowling RH. Gallbladder emptying patterns in response to a normal meal in healthy subjects and patients with gallstones: ultrasound study. *Gut* 1991; **32**: 1406-1411
- 22 **Fisher RS**, Stelzer F, Rock E. Abnormal gallbladder emptying in patients with gallstones. *Dig Dis Sci* 1982; **27**: 1019-1024
- 23 **Shaffer EA**, McOrmond P, Duggan H. Quantitative cholecintigraphy: assessment of gallbladder filling and emptying and duodenogastric reflux. *Gastroenterology* 1980; **79**: 899-906
- 24 **Van Berge Henegouwen GP**, Hofman AF. Nocturnal gallbladder storage and emptying in gallstone patients and healthy subjects. *Gastroenterology* 1978; **75**: 879-885
- 25 **Maudgal DP**, Kupfer RM, Zentler-Munro PL. Postprandial gallbladder emptying in patients with gallstones. *BMJ* 1980; **280**: 141-143
- 26 **van Erpecum KJ**, van Berg Henegouwen GP, Stolk MFG. Fasting gallbladder volume, postprandial emptying and cholecystokinin release in gallstone patients and normal subjects. *J Hepatol* 1992; **14**: 194-202
- 27 **Heaton KW**, Braddon FE, Mountford RA. Symptomatic and silent gall stones in community. *Gut* 1991; **32**: 316-332
- 28 **Debas HT**, Yamagishi T. Evidence for a pyloro-cholecystic reflex for gallbladder contraction. *Ann Surg* 1979; **190**: 170-175
- 29 **Froehlich F**, Gonvers JJ, Fried M. Role of nutrient fat and cholecystokinin in regulation of gallbladder emptying in man. *Dig Dis Sci* 1995; **40**: 529-533
- 30 **Yamamura T**, Takahashi T, Kusunoki M. Gallbladder dynamics and plasma cholecystokinin responses after meals, oral water, or sham feeding in healthy subjects. *Am J Med Sci* 1988; **295**: 102-107

Edited by Wang XL Proofread by Zhu LH



• CLINICAL RESEARCH •

# Relationship between inducible nitric oxide synthase expression and angiogenesis in primary gallbladder carcinoma tissue

Xin-Jie Niu, Zuo-Ren Wang, Sheng-Li Wu, Zhi-Min Geng, Yun-Feng Zhang, Xing-Lei Qing

**Xin-Jie Niu, Zuo-Ren Wang, Sheng-Li Wu, Zhi-Min Geng, Yun-Feng Zhang, Xing-Lei Qing**, Department of Hepatobiliary Surgery, First Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China

**Correspondence to:** Dr. Xin-Jie Niu, Department of Hepatobiliary Surgery, First Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China. niuxinjie@email.com

**Telephone:** +86-29-85324009 **Fax:** +86-29-85324009

**Received:** 2003-05-11 **Accepted:** 2003-06-07

## Abstract

**AIM:** To explore the relationship between angiogenesis and biological behaviors of primary gallbladder carcinoma (PGBC), the relationship between the expression of inducible nitric oxide synthase (iNOS) and biological behaviors of PGBC and its relationship with the expression of iNOS and angiogenesis of PGBC.

**METHODS:** The expression of iNOS and micro-vessel density (MVD) were assessed by immunohistochemical method and image analysis system in 40 specimens of PGBC and in 8 specimens of normal gallbladder. The immunostaining results and related clinicopathologic materials were analyzed by statistical methods.

**RESULTS:** MVD in PGBC was significantly higher than that in normal gallbladder tissue ( $46 \pm 14$  vs  $14 \pm 6$ ,  $P < 0.05$ ), and was not related with age, gender, tumor size and histological type. MVD of poorly and undifferentiated tumor tissues was higher than that of moderately-differentiated and well-differentiated tumor tissues ( $52 \pm 9$  vs  $43 \pm 9$  vs  $33 \pm 6$ ,  $P < 0.01$ ). MVD of Nevin IV and V stages was higher than that of Nevin I, II and III stages ( $52 \pm 8$  vs  $37 \pm 13$ ,  $P < 0.01$ ). MVD of cases with lymphatic or liver metastasis was significantly higher than that without liver metastasis ( $55 \pm 6$  vs  $42 \pm 10$ ,  $P < 0.05$ ) or lymphatic metastasis ( $53 \pm 8$  vs  $38 \pm 8$ ,  $P < 0.01$ ). The positive level index (PLI) of iNOS in PGBC was  $0.435 \pm 0.134$ , and was not related with age, gender, tumor size, histological type, differentiation and clinical stage of PGBC. The PLI of iNOS in cases with lymphatic metastasis was higher than that without lymphatic metastasis ( $0.573 \pm 0.078$  vs  $0.367 \pm 0.064$ ,  $P < 0.01$ ). The PLI of iNOS in cases with liver metastasis was higher than that without liver metastasis ( $0.533 \pm 0.067$  vs  $0.424 \pm 0.084$ ,  $P < 0.05$ ). There was a significant correlation between PLI of iNOS and MVD in PGBC ( $P < 0.05$ ).

**CONCLUSION:** Angiogenesis of PGBC is significantly related to the biological behaviors of PGBC. The expression of iNOS is related to the biological behaviors of PGBC. The detection of MVD and the expression of iNOS in PGBC can be used as parameters to determine the degree of malignancy and prognosis.

Niu XJ, Wang ZR, Wu SL, Geng ZM, Zhang YF, Qing XL. Relationship between inducible nitric oxide synthase expression and angiogenesis in primary gallbladder carcinoma tissue. *World J Gastroenterol* 2004; 10(5): 725-728

<http://www.wjgnet.com/1007-9327/10/725.asp>

## INTRODUCTION

PGBC is a kind of malignant neoplasm with poor prognosis, and accounts for 1.5% of digestive carcinomas<sup>[1]</sup>. It is difficult to be diagnosed in its early stage. Recent studies have shown its morbidity is gradually increasing<sup>[2]</sup>. Tumor growth is a multistage process and tumor angiogenesis is one of the key steps in tumor growth, infiltration and metastasis. In many tumors, the expression of iNOS is strong<sup>[3]</sup>. NO is synthesized from the amino acid L-arginine by iNOS and has many biological functions closely related with carcinogenesis and development of carcinomas, especially with tumor angiogenesis<sup>[4]</sup>. At present, the study about angiogenesis of PGBC is few and the relationship between angiogenesis and iNOS of PGBC has not been reported. In the present study the expressions of iNOS and MVD of 40 PGBCs and 8 normal gallbladders were investigated by immunohistochemistry and image analysis methods. The clinicopathologic indexes, the relationship between expression of iNOS and angiogenesis of PGBC and significance of the expression of iNOS were discussed.

## MATERIALS AND METHODS

### Pathological materials

Forty specimens of PGBC were collected between 1996 and 2002 in the Department of Hepatobiliary Surgery, First Hospital of Xi'an Jiaotong University. No treatment was given before operation. Specimens were fixed with formaldehyde and embedded with paraffin. The histological grading of tumor was done on hematoxylin-eosin stained sections. There were 11 males and 29 females, their mean age of patients was 59 years old. Among the 35 adenocarcinomas, 31 belonged to differentiated adenocarcinomas, 2 mucinous adenocarcinomas and 2 undifferentiated adenocarcinomas. There were 2 adenosquamous carcinomas, 1 adenoacanthoma, 1 sarcoma carcinoma and 1 neuroendocrine carcinoma. Eighteen tumours had a size  $\geq 3$  cm, 22 had a size  $< 3$  cm. Seven were well differentiated carcinomas, 14 moderately differentiated, 16 poorly differentiated and 3 undifferentiated. Twenty cases had metastases in lymph nodes, 11 in liver. Eight normal gallbladders were studied as control.

### Immunohistochemistry

Tissue samples were fixed in 10g/L neutral-buffered formaldehyde, embedded in paraffin, sectioned (4  $\mu$ m thick), and deparaffinized. Slides were immersed first in 3 mL/L  $H_2O_2$  at room temperature for 10 min to get rid of the activity of endogenous peroxidase. Then slides were digested with 1 g/L trypsin for 10 min. The slides were washed with distilled water, soaked in PBS for 5 min, then put into microwave at 95  $^{\circ}$ C for 8 min to repair the antigen. Then slides were immersed in goat serum (1.5%) at room temperature for 40 min to block endogenous nonspecific binding sites. Immunostaining was performed with the primary rabbit polyclonal IgG specific for iNOS (dilution, 1:50; Santa Cruz Biotechnology, USA) at room temperature for 2 h. The primary rabbit polyclonal IgG specific for factor VIII-related antigen (dilution, 1:100; Sigma Biotechnology, USA) was used. Then a biotinylated secondary antirabbit antibody

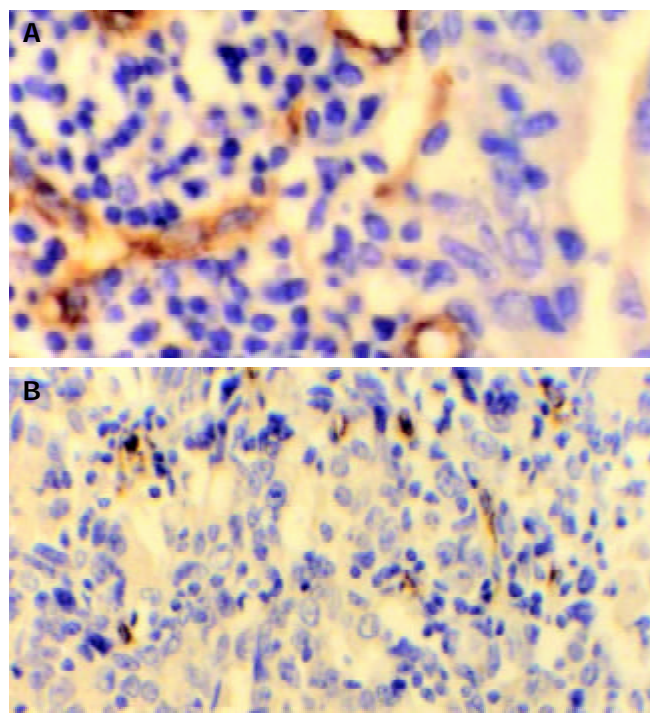
(BOSHIDE Biotechnology, Wuhan, China) diluted 1:200 in PBS was applied on the sections for 20 min, followed by the streptavidin-biotin-peroxidase complex (dilution, 1:200; BOSHIDE Biotechnology, Wuhan, China) for 20 min. The color was developed by diaminobenzidine (DAB). The sections were counter-stained with hematoxylin, dehydrated, made transparent, covered and observed. Primary antibody replaced by PBS was used as a negative control. For positive controls, hemangioma was stained for factor VIII-related antigen and gastric carcinoma for iNOS.

#### Determination of iNOS

Under light microscope the positive cells were stained as brownish yellow in cytoplasm. Determination of iNOS was performed by an image analysis system (IBAS System, Kontron Eledtronik, Germany). According to guide system set operation was made for determination of optical density. The average optical density (AOD) of 100-200 iNOS positive cells was randomly measured with  $\times 20$  objective, and the average percentage of positive cells (APCP) was obtained by measuring positive cells and total tumor cells in random 10 high power fields (HPFs) with  $\times 40$  objective. The positive level index (PLI) was calculated according to the following formula:  $PLI = APCP \times AOD$ .

#### Quantification of angiogenesis

Determination of MVD was based upon the method reported by Marrogi *et al*<sup>[6]</sup>. An all-round observation was first made with  $\times 10$  objective, then five areas with the highest density of micro-vessels (hot spots) were selected, and the amount of micro-vessels was counted with  $\times 20$  objective (any endothelial cell or endothelial cluster close to tumor cells and connective tissue around tumor cells, which was stained brownish yellow were considered as a single, countable micro-vessel). MVD was counted from an average of five HPFs with  $\times 20$  objective (Figure 1).



**Figure 1** Distribution of MVD in PGBC tissue, SABC  $\times 200$ . A: high MVD, B: low MVD.

#### Statistical analysis

SPSS for Windows (10.0 edition) was used for statistical analysis. All data were expressed as mean  $\pm$  SD. Statistical

differences were evaluated using the unpaired *t* test, *F* test and *q* test. Analysis of linear correlation was used to discuss the relationship between expressions of iNOS and MVD. A *P* value less than 0.05 was considered statistically significant.

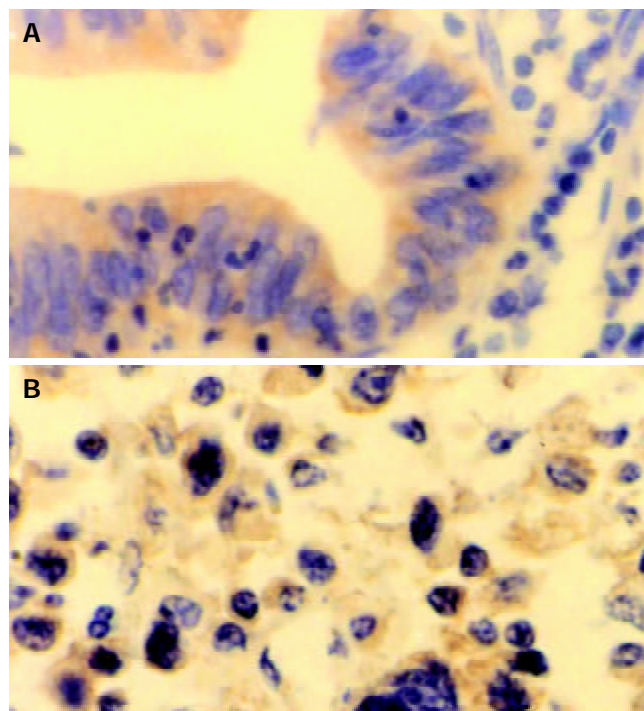
## RESULTS

#### MVD and PGBC

Through the stains by polyclonal IgG specific for factor VIII-related antigen, micro-vessels could be identified. The distribution of micro-vessels in PGBC was not even, which was irregular in morphology and differed greatly in quantity (Figure 1). MVD was the highest at the margin of tumor tissues. MVD ( $46 \pm 14$ ) in PGBC was significantly higher than that ( $14 \pm 6$ ) in normal gallbladder tissue ( $P < 0.05$ ). MVD in PGBC was not related with age, gender, tumor size and histological type. MVD in poorly and undifferentiated tumor tissues was higher than that of moderately differentiated and well-differentiated tumor tissues ( $P < 0.01$ ). MVD of Nevin IV and V stages was higher than that of Nevin I, II and III stages ( $P < 0.01$ ). MVD of cases with lymphatic or liver metastasis was significantly higher than that without liver metastasis ( $P < 0.05$ ) or lymphatic metastasis ( $P < 0.01$ , Table 1).

#### iNOS and PGBC

The expression of iNOS in normal gallbladder epithelial cells was negative and occasionally positive in stromal cells. In tissue of PGBC, iNOS was mainly expressed in cytoplasm and few in inflammatory cells around the tumor cells (Figure 2). The PLI of iNOS in 40 PGBCs was  $0.435 \pm 0.134$ . It was not related with age, gender, tumor size, histological type, differentiation and clinical stage of PGBC. The PLI of iNOS in cases with lymphatic metastasis was higher than that without lymphatic metastasis ( $P < 0.01$ ). The PLI of iNOS in cases with liver metastasis was higher than that without liver metastasis ( $P < 0.05$ , Table 1).



**Figure 2** Positive expression of iNOS in PGBC tissues SABC  $\times 400$ . A: well-differentiated, B: poorly-differentiated.

#### iNOS and angiogenesis of PGBC

By serial analysis of sections, high MVD was observed in

areas of high iNOS expression. Linear correlation analysis showed that PLI of iNOS was positively correlated with MVD ( $r=0.4021$ ,  $P<0.05$ ). This demonstrated that expression of iNOS could influence MVD and blood supply of tumor.

**Table 1** Relationship between MVD, PLI and clinicopathologic indexes

Group	n	MVD	PLI
Age <58yr	17	43±14	0.340±0.048
≥58yr	23	45±11	0.371±0.061
Male	11	46±11	0.376±0.056
Female	29	44±11	0.421±0.073
Size ≥3 cm	18	42±11	0.365±0.077
<3 cm	22	46±10	0.434±0.097
Adenocarcinoma	35	45±12	0.473±0.086
Adeno-squamouscarcinoma	2	41±3	0.391±0.131
Others	3	47±6	0.447±0.025
Differentiation well	7	33±6	0.397±0.089
Moderate	14	43±9 <sup>a</sup>	0.413±0.072
Poor & Non	19	52±9 <sup>ac</sup>	0.453±0.113
Nevin stages I, II, III	18	37±13	0.410±0.092
IV, V	22	52±8 <sup>b</sup>	0.459±0.088
Lymphatic Metastasis +	20	53±8	0.573±0.078
-	20	38±8 <sup>d</sup>	0.367±0.064 <sup>f</sup>
Liver Metastasis +	11	55±6	0.533±0.067
-	29	42±10 <sup>e</sup>	0.424±0.084 <sup>g</sup>

<sup>a</sup> $P<0.05$ , vs Well. <sup>c</sup> $P<0.05$ , vs Moderate. <sup>b</sup> $P<0.01$ , vs I, II, III Stages.

<sup>d,f</sup> $P<0.01$ , vs Positive. <sup>e,g</sup> $P<0.05$ , vs Positive.

## DISCUSSION

The formation of new blood vessels known as angiogenesis induced by tumor cells is a critical determinant of tumor progression<sup>[5-10]</sup>. Unlike normal blood vessels, tumor blood vessels are not mature vessels which are chaotic, irregular and leaky. Studies showed that proliferation, infiltration and metastasis of solid tumor were closely related with tumor angiogenesis<sup>[11-16]</sup>. In our study we found that the distribution of micro-vessels in PGBC was not even, but irregular in morphology and its quantity differed greatly. MVD was the highest at the margin of tumor tissues. In tumor tissues MVD was higher than that in normal tissues. Our study showed that MVD in PGBC was significantly higher than that in normal gallbladder tissue. Research on mammary cancer showed that MVD was increased in patients with distant metastasis compared with those without distant metastasis<sup>[17]</sup>. The risk of metastasis would increase by 159% per increase of 10 micro-vessels<sup>[18]</sup>. This study found that MVD in cases with lymphatic or liver metastasis was significantly higher than that without liver or lymphatic metastasis, which was consistent with other researchers<sup>[19-22]</sup>. It is generally believed that invasion of tumor cells from tumor tissue with rich vasculature increases and the risk of lymphatic metastasis is increased by invasion of veno-lymphatic anastomosis and lymphatic vessels accompanying blood capillaries.

Calcium-independent iNOS was expressed in macrophages, neutrophils, hepatocytes, cardiac myocytes, chondrocytes, and many other cell types<sup>[23-26]</sup>. It was mainly induced by cytokines and could generate locally high concentrations of NO for a prolonged period of time and play a variety of regulatory functions *in vivo*<sup>[27,28]</sup>. In many studies, iNOS positivity was predominantly found in tumor cells<sup>[29,30]</sup>, but in another study a relatively high iNOS immunoreactivity was noted in stromal cells<sup>[31]</sup>. Our study showed that in PGBC iNOS was mainly expressed in cytoplasm of tumor cells and in few inflammatory

cells around the tumor cells. Vakkala *et al*<sup>[32]</sup> showed that iNOS positivity was observed in mammary cancer cells in 46.5% *in situ* carcinomas and 58.8% invasive carcinomas. Expression of iNOS was related with differentiation of carcinomas *in situ*. In this study PLI of iNOS was not related to age, gender, tumor size, histological type, differentiation and clinical stage of PGBC. The PLI of iNOS in cases with lymphatic metastasis was higher than that without lymphatic metastasis and the PLI of iNOS in cases with liver metastasis was higher than that without liver metastasis. The effects of NO could be tumor promoting or tumor suppressing. High concentrations of NO (umol/L) could be cytotoxic, whereas low concentration (nmol/g or pmol/g) might even protect some cell types from damage and apoptosis<sup>[33]</sup>. During the initiation of tumor growth, natural killer cells and macrophages could kill tumor cells by a NO-mediated mechanism<sup>[34]</sup>. However, NO might also suppress antitumor effect, promote tumor angiogenesis and blood flow in tumor neovasculature, and enhance tumor growth, invasion, and metastasis<sup>[35]</sup>. Jenkins *et al*<sup>[36]</sup> engineered gene of iNOS into adenocarcinoma cell line DLD-1 to get iNOS-19 subclone which generated NO continuously, and found that in nude mice tumors from iNOS-19 subclone cells grew faster than those derived from wild-type cells and were much markedly vascularized and had a stronger ability to invade. Our conclusion is that NO produced by iNOS in PGBC is in low concentration and can promote tumor angiogenesis, invasion and metastasis of PGBC.

## REFERENCES

- 1 **Ishikawa T**, Horimi T, Shima Y, Okabayashi T, Nishioka Y, Hamada M, Ichikawa J, Tsuji A, Takamatsu M, Morita S. Evaluation of aggressive surgical treatment for advanced carcinoma of the gallbladder. *J Hepatobiliary Pancreat Surg* 2003; **10**: 233-238
- 2 **Yamamoto T**, Uki K, Takeuchi K, Nagashima N, Honjo H, Sakurai N, Okuda C, Watanabe G, Mori M, Kuyama Y. Early gallbladder carcinoma associated with primary sclerosing cholangitis and ulcerative colitis. *J Gastroenterol* 2003; **38**: 704-706
- 3 **Kvasnicka T**. NO (nitric oxide) and its significance in regulation of vascular homeostasis. *Vnitr Lek* 2003; **49**: 291-296
- 4 **Esken FA**. Angiogenesis inhibitors in clinical development; where are we now and where are we going? *Br J Cancer* 2004; **90**: 1-7
- 5 **Detmar M**. Tumor angiogenesis. *J Invest Dermatol Symp Proc* 2000; **5**: 20-23
- 6 **Marrogi AJ**, Travis WD, Welsh JA, Khan MA, Rahim H, Tazelaar H, Pairolero P, Trastek V, Jett J, Caporaso NE, Liotta LA, Harris CC. Nitric oxide synthase, cyclooxygenase 2, and vascular endothelial growth factor in the angiogenesis of non-small cell lung carcinoma. *Clin Cancer Res* 2000; **6**: 4739-4744
- 7 **Gupta MK**, Qin RY. Mechanism and its regulation of tumor-induced angiogenesis. *World J Gastroenterol* 2003; **9**: 1144-1155
- 8 **Abdulkadir SA**, Carvalho GF, Kaleem Z, Kisiel W, Humphrey PA, Catalona WJ, Mibrandt J. Tissue factor expression and angiogenesis in human prostate carcinoma. *Hum Pathol* 2000; **31**: 443-447
- 9 **Forootan SS**, Ke Y, Jones AS, Helliwell TR. Basic fibroblast growth factor and angiogenesis in squamous carcinoma of the tongue. *Oral Oncol* 2000; **36**: 437-443
- 10 **Yoshikawa T**, Yanoma S, Tsuburaya A, Kobayashi O, Sairenji M, Motohashi H, Noguchi Y. Angiogenesis inhibitor, TNP-470, suppresses growth of peritoneal disseminating foci. *Hepatogastroenterology* 2000; **47**: 298-302
- 11 **Erenoglu C**, Akin ML, Uluutku H, Tezcan L, Yildirim S, Batkin A. Angiogenesis predicts poor prognosis in gastric carcinoma. *Dig Surg* 2000; **17**: 581-586
- 12 **Shimoyama S**, Kaminishi M. Increased angiogenin expression in gastric cancer correlated with cancer progression. *J Cancer Res Clin Oncol* 2000; **126**: 468-474
- 13 **Xiong B**, Gong LL, Zhang F, Hu MB, Yuan HY. TGF  $\beta$ 1 expression and angiogenesis in colorectal cancer tissue. *World J Gastroenterol* 2002; **8**: 496-498
- 14 **Gu ZP**, Wang YJ, Li JG, Zhou YA. VEGF165 antisense RNA sup-

- presses oncogenic properties of human esophageal squamous cell carcinoma. *World J Gastroenterol* 2002; **8**: 44-48
- 15 **Li HX**, Chang XM, Song ZJ, He SX. Correlation between expression of cyclooxygenase-2 and angiogenesis in human gastric adenocarcinoma. *World J Gastroenterol* 2003; **9**: 674-677
- 16 **Coomber BL**, Yu JL, Fathers KE, Plumb C, Rak JW. Angiogenesis and the role of epigenetics in metastasis. *Clin Exp Metastasis* 2003; **20**: 215-227
- 17 **Toivonen P**, Makitie T, Kujala E, Kivela T. Microcirculation and tumor-infiltrating macrophages in choroidal and ciliary body melanoma and corresponding metastases. *Invest Ophthalmol Vis Sci* 2004; **45**: 1-6
- 18 **Wong YK**, Liu CJ, Kwan PC, Chao SY. Microvascular density and vascular endothelial growth factor immunoreactivity as predictors of regional lymph node metastasis from betel-associated oral squamous cell carcinoma. *J Oral Maxillofac Surg* 2003; **61**: 1257-1262
- 19 **Sugawara Y**, Makuuchi M, Harihara Y, Noie T, Inoue K, Kubota K, Takayama T. Tumor angiogenesis in gallbladder carcinoma. *Hepatogastroenterology* 1999; **46**: 1682-1686
- 20 **Nakashima T**, Kondoh S, Kitoh H, Ozawa H, Okita S, Harada T, Shiraishi K, Ryozaawa S, Okita K. Vascular endothelial growth factor-C expression in human gallbladder cancer and its relationship to lymph node metastasis. *Int J Mol Med* 2003; **11**: 33-39
- 21 **Giatromanolaki A**, Sivridis E, Simopoulos C, Polychronidis A, Gatter KC, Harris AL, Koukourakis MI. Thymidine phosphorylase expression in gallbladder adenocarcinomas. *Int J Surg Pathol* 2002; **10**: 181-188
- 22 **Giatromanolaki A**, Sivridis E, Koukourakis MI, Polychronidis A, Simopoulos C. Prognostic role of angiogenesis in operable carcinoma of the gallbladder. *Am J Clin Oncol* 2002; **25**: 38-41
- 23 **Hunter RP**. Nitric oxide, inducible nitric oxide synthase and inflammation in veterinary medicine. *Anim Health Res Rev* 2002; **3**: 119-133
- 24 **Ortiz PA**, Garvin JL. Cardiovascular and renal control in NOS-deficient mouse models. *Am J Physiol Regul Integr Comp Physiol* 2003; **284**: R628-638
- 25 **Qin JM**, Zhang YD. Intestinal expressions of eNOSmRNA and iNOSmRNA in rats with acute liver failure. *World J Gastroenterol* 2001; **7**: 652-656
- 26 **Dong WG**, Mei Q, Yu JP, Xu JM, Xiang L, Xu Y. Effects of melatonin on the expression of iNOS and COX-2 in rat models of colitis. *World J Gastroenterol* 2003; **9**: 1307-1311
- 27 **Beauregard C**, Brandt PC, Chiou GC. Induction of nitric oxide synthase and over-production of nitric oxide by interleukin-1beta in cultured lacrimal gland acinar cells. *Exp Eye Res* 2003; **77**: 109-114
- 28 **Zhou JL**, Jin GH, Yi YL, Zhang JL, Huang XL. Role of nitric oxide and peroxynitrite anion in lung injury induced by intestinal ischemia-reperfusion in rats. *World J Gastroenterol* 2003; **9**: 1318-1322
- 29 **Lala PK**, Orlucevic A. Role of nitric oxide in tumor progression: lessons from experimental tumors. *Cancer Metastasis Rev* 1998; **17**: 91-106
- 30 **Kojima M**, Morisaki T, Tsukahara Y, Uchiyama A, Matsunari Y, Mibu R, Tanaka M. Nitric oxide synthase expression and nitric oxide production in human colon carcinoma tissue. *J Surg Oncol* 1999; **70**: 222-229
- 31 **Aaltoma SH**, Lipponen PK, Kosma VM. Inducible nitric oxide synthase (iNOS) expression and its prognostic value in prostate cancer. *Anticancer Res* 2001; **21**: 3101-3106
- 32 **Vakkala M**, Kahlos K, Lakari E, Paakko P, Kinnula V, Soini Y. Inducible nitric oxide synthase expression, apoptosis, and angiogenesis in *in situ* and invasive breast carcinoma. *Clin Cancer Res* 2000; **6**: 2408-2416
- 33 **Ambis S**, Hussain SP, Harris CC. Interactive effects of nitric oxide and the p53 tumor suppressor gene in carcinogenesis and tumor progression. *FASEB J* 1997; **11**: 443-448
- 34 **Wink DA**, Vodovotz Y, Laval J, Laval F, Dewhirst MW, Mitchell JB. The multifaceted roles of nitric oxide in cancer. *Carcinogenesis* 1998; **19**: 711-721
- 35 **Song ZJ**, Gong P, Wu YE. Relationship between the expression of iNOS, VEGF, tumor angiogenesis and gastric cancer. *World J Gastroenterol* 2002; **8**: 591-595
- 36 **Jenkins DC**, Charles IG, Thomsen LL, Moss DW, Holmes LS, Baylis SA, Rhodes P, Westmore K, Emson PC, Moncada S. Roles of nitric oxide in tumor growth. *Proc Natl Acad Sci U S A* 1995; **92**: 4392-4396

Edited by Xu JY and Wang XL

# Effects of low molecular weight heparin on platelet surface P-selectin expression and serum interleukin-8 production in rats with trinitrobenzene sulphonic acid-induced colitis

Bing Xia, Hong Han, Ke-Jian Zhang, Jin Li, Guang-Song Guo, Ling-Ling Gong, Xian-Chang Zeng, Jun-Yan Liu

**Bing Xia, Hong Han, Ke-Jian Zhang, Jin Li, Xian-Chang Zeng,**  
Department of Internal Medicine, Zhongnan Hospital, Wuhan University, Wuhan 430071, Hubei Province, China

**Guang-Song Guo, Ling-Ling Gong,** Department of Pathology, Zhongnan Hospital, Wuhan University, Wuhan 430071, Hubei Province, China

**Jun-Yan Liu,** Department of Immunology, Medical School, Wuhan University, Wuhan 430071, Hubei Province, China

**Supported by** the Hubei Provincial Natural Science Foundation, No. 2000J047

**Correspondence to:** Professor Bing Xia, Department of Internal Medicine, Zhongnan Hospital, Wuhan University, Wuhan 430071, Hubei Province, China. bingxia@public.wh.hb.cn

**Telephone:** +86-27-67812985 **Fax:** +86-27-87307622

**Received:** 2003-07-10 **Accepted:** 2003-08-18

## Abstract

**AIM:** To observe the effects of low molecular weight heparin (LMWH) on platelet surface P-selectin expression and serum interleukin-8 production in rats with trinitrobenzene sulphonic acid (TNBS) induced colitis.

**METHODS:** Colitis was induced in female Sprague-Dawley rats by colonic administration of 2, 4, 6-TNBS. LMWH, a dalteparin (150 U/kg, 300 U/kg) was subcutaneously administered one hour before induction of colitis and went on once a day for 6 days. Then a half dose was given for the next 7 days. Control animals received the same volume of normal saline once a day for 14 days after treated by TNBS. Animals were sacrificed at 24 h, days 7 and 14 after induction of colitis. The colon was excised for the evaluation of macroscopic and histological findings and TNF- $\alpha$  immunohistochemical assay. Platelet surface P-selectin expression was determined by radioimmunoassay and serum IL-8 production was assayed by ELISA method.

**RESULTS:** LMWH treatment in a dose of 300 U/kg for 14 days significantly improved colonic inflammation by histological examination. Serum IL-8 production in the 300 U/kg treatment group was more significantly decreased at day 14 than that at 24 h ( $P < 0.05$ ). However, platelet surface P-selectin expression and TNF- $\alpha$  staining in colonic tissue were not significantly different among the three groups.

**CONCLUSION:** LMWH has an anti-inflammatory effect on TNBS induced colitis in rats. The effect is possibly related to inhibition of proinflammatory cytokine IL-8, but not involved platelet surface P-selectin expression.

Xia B, Han H, Zhang KJ, Li J, Guo GS, Gong LL, Zeng XC, Liu JY. Effects of low molecular weight heparin on platelet surface P-selectin expression and serum interleukin-8 production in rats with trinitrobenzene sulphonic acid-induced colitis. *World J Gastroenterol* 2004; 10(5): 729-732  
<http://www.wjgnet.com/1007-9327/10/729.asp>

## INTRODUCTION

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease (CD), both of which are chronic non-specific intestinal inflammation with unknown etiology. Several studies have shown that IBD exists a hypercoagulant state in active period of the disease and may have microvascular thrombosis in the wall of intestine<sup>[1-8]</sup>. An abnormal platelet activity has been reported in patients with UC and CD, and plays an important role in inflammation aggravation<sup>[9-12]</sup>. P-selectin is a membrane glycoprotein, which is expressed on activated platelets and endothelial cells, promotes leukocyte adhesion and migration as well as inflammatory cytokine production. IL-8 is a key proinflammatory cytokine and its production is increased in activated platelets. An up-regulation of platelet IL-8 receptors in patients with IBD has been reported<sup>[11]</sup>.

Heparin is a glycoaminoglycan formed by sulphatedoligosaccharides and varies in the length of polymeric units and therefore has different molecular weights. Low molecular weight heparin (LMWH) is made by partial hydrolysis or enzymatic degradation of unfractionated heparin. Heparin and LMWH prevent the process of blood coagulation and have a natural anti-thrombin effect. In recent years several studies have shown that heparin and LMWH have an obvious anti-inflammatory activity in addition to its traditional anticoagulant effects<sup>[9,13,14]</sup>. In animal model heparin disaccharides inhibited TNF- $\alpha$  production by macrophages and decreased immune inflammation<sup>[15]</sup>. Heparin accelerated the healing process of mucosa in colitis in several clinical studies and had anti-inflammatory effects<sup>[16-21]</sup>. Therefore, administration of heparin can afford both anti-inflammatory and anticoagulant effects. The mechanisms of anti-inflammation of heparin are unknown. The limited studies demonstrated that it was possibly associated with the increase of several growth factors and the decrease of nitric oxide synthesis (NOS) and myeloperoxidase<sup>[9,13,14,22,23]</sup>. The effects of heparin on platelet surface P-selectin and IL-8 have not been studied. The present study was designed to observe the effects of LMWH on platelet surface P-selectin expression, serum IL-8 production, and TNF- $\alpha$  expression in mucosa of rats with trinitrobenzene sulphonic acid (TNBS)-induced colitis, and to clarify the anti-inflammatory mechanisms of LMWH in the treatment of colitis.

## MATERIALS AND METHODS

### Animals

Female Sprague-Dawley rats weighing 200-250 g were used in the study. The animals were fasted for 24 h before the experiment and allowed food and water *ad libitum* after induction of colitis. The study was approved by the Ethic Committee of Wuhan University Medical School.

### Induction of TNBS-induced colitis

Colitis was induced by a method of hapten-induced colonic inflammation as previously described<sup>[24]</sup>. Rats were anaesthetized by intraperitoneal administration of 100g/L urethane. A small



volume of 2, 4, 6-TNBS (Sigma Company) was dissolved in 50% ethanol to a final concentration of 100 mg/mL, and 0.3 mL of TNBS solution was intracolonicly administrated with a polypropylene catheter by inserting 8 cm via the anal canal. Rats in a group were given 150 U/kg LMWH (dalteparin made by Pharmacia & Upjohn Company) subcutaneously 1 h before induction of colitis, and went on once a day for 6 d. Then a half dose of LMWH was given for the next 7 d. Treatment of the LMWH 300 U/kg group was the same as the 150 U/kg group, but the dose of LMWH was as high as 300 U/kg. Control animals received the same volume of normal saline once a day instead of LMWH after treated by TNBS.

Animals in each group were sacrificed at 24 h, d 7 and 14 after induction of colitis. The colon was isolated and a segment of colon was excised for the evaluation of macroscopic and histological findings and also for TNF- $\alpha$  immunohistochemical assay. A blood sample was drawn from heart of the rats for determination of platelet surface P-selectin and serum IL-8 before the rats were sacrificed.

### Macroscopic evaluation of colonic damage

Macroscopic evaluation of colonic damage was conducted by two authors (HH, LLG) and mucosal hyperemia, ulcers, inflammatory exudation and bleeding were recorded. The most damaged site of the colon was chosen as the part for histological studies. For control group the colon at 8 cm above anus was excised for histological studies.

### Histological studies of colonic damage

For histological examination, formalin-fixed tissues were embedded in paraffin and 5  $\mu$ m -thick sections were stained with hematoxylin and eosin, and evaluated under light microscope by a pathologist (GSG) blinded to the experimental protocol. Colonic damage was assessed by the grades described by Fedorak *et al*<sup>[25]</sup>. Mucosal ulceration: 0: no-ulceration; 1: focal ulceration; 2: multifocal-ulceration; 3: diffuse ulceration. Depth of injury was graded as follows: 0: no injury; 1: mucosal involvement only; 2: mucosal and submucosal involvement; 3: transmural involvement. The ulceration and depth of injury grades were scored and put together as a result ranging between the minimum of 0 and maximum of 6.

### Determination of platelet surface P-selectin molecules

Platelet surface P-selectin molecules were detected by radioimmunoassay. The kit was purchased from the Institute of Thrombosis and Homeostasis of Suzhou University, China. Briefly, 2 mL of anti-coagulated blood was taken from the heart of rats and platelets were counted under microscope. The blood was fixed by 2g/L glutaraldehyde in PBS solution at room temperature for 30 min and stored at 4 °C for determination of platelet surface P-selectin. The fixed blood with  $2.5 \times 10^6$  platelets was divided into 3 tubes and washed with 1 mL of 0.01mol/L PBS, pH 7.4, per tube and centrifuged at 2 500 rpm for 10 min. Supernatants were removed. 50  $\mu$ L of SZ-51mab labeled with  $^{125}$ I was added into the reaction tubes and mixed fully. 15  $\mu$ L of SZ-51mab without  $^{125}$ I was added into control tubes. Each tube was incubated at 37 °C, washed 3 times with PBS, and centrifuged at 3 000 r/min for 10 min. Cpm of precipitations in each tube was measured by a  $\gamma$  reader.

### Determination of serum IL-8 production

Serum IL-8 production was assayed by ELISA sandwich method. The IL-8 ELISA kit was purchased from the Institute of Immunology of the Fourth Military Medical University, China. Briefly, 96-well plates were precoated with monoclonal antibody specific to human IL-8. Serum samples, negative control and diluted IL-8 standard markers were added into the

plates. The serum samples were detected according to the procedures described in the protocol.  $A_{410}$  value was read by the ELISA reader.

### Determination of TNF- $\alpha$ in colonic mucosa

Expression of TNF- $\alpha$  in colonic mucosa was assessed by immunohistochemistry. The reagents of TNF- $\alpha$  were purchased from Beijing Zhongshan Biotechnology, China. Positive expression of TNF- $\alpha$  was brown deposited granules in the plasma of neutrophils, monocytes and lymphocytes. The grades of expression of TNF- $\alpha$  in mucosa were classified as follows: 0: no staining; 1: a few maple granules, or a few fine brown granules, not exceeding the 1/4 total area of cytoplasm; 2: uniformity maple in the whole cytoplasm or wide brown granules in the cytoplasm, not exceeding the 1/2 area of cytoplasm; 3: the cytoplasm was full of brown granules with a lower density; 4: the total cytoplasm was full of crassitude dark brown granules, and covered whole nuclei of the cell. One hundred cells were counted under oil microscope, positive cells were recorded and positive cell rate was calculated. The scores of expression of TNF- $\alpha$  were calculated by sum of each grade multiplying its positive cell rate.

### Statistical analysis

Data were expressed as mean $\pm$ SD. Data in different groups were analyzed by ANOVA and post multiple tests (Tukey-Kramer or Student-Newman-Keuls). A *P* value less than 0.05 was considered statistically significant.

## RESULTS

### Macroscopic evaluation of TNBS-induced colitis

Control animals subjected to intracolonic administration of TNBS in 500mL ethanol showed colonic mucosal injury with ulceration. Signs of the mucosal damage were monitored for 14 d. As early as 24 h after TNBS treatment, colonic mucosa was shown to have hyperemia, congestion, erosion, hemorrhagic ulcerations in the injured site. The damage was still maintained at d 7 and appeared to be multiple ulcerations and partial epithelial necrosis. On d 14, the colonic ulceration still existed. In both doses of LMWH treated groups, mucosal hyperemia, congestion and ulcerations in the injured site were slighter than those in control group. However, mucosal hemorrhage in both LMWH treated groups was severer than that in control group at 24 h, but was resolved on d 7 and 14, respectively.

### Histological examination of TNBS-induced colitis

In control group a large number of neutrophils, monocytes and eosinophils infiltrated in mucosa and submucosa at 24 h and the damage reached peak on d 7. As shown in Table 1, the histological grades according to Fedorak *et al*<sup>[25]</sup> were greatly higher on d 7 compared with those at 24 h (*P*<0.01). On d 14, the damage of colon was mild, but still had ulceration, chronic inflammatory cell infiltration, vesiculitis and granulation tissue formation. In contrast, in both 150 U/kg and 300 U/kg LMWH treated groups colonic damage was mild. Table 1 shows that the histological injury grades in the 300 U/kg LMWH group were decreased at 24 h and on d 7 after treatment, and much obviously on day 14 compared with the control group (*P*<0.01).

### Expression of TNF- $\alpha$ in colonic mucosa of TNBS-induced colitis

As shown in Table 2, the scores of expression of TNF- $\alpha$  in mucosa were greatly higher in the first 24 h and decreased on d 7 and 14 after induction of colitis, but there were no



significant differences among the three groups except a difference at 24 h between 300 U/kg LMWH treatment group and 150 U/kg LMWH treatment group.

**Table 1** Effect of low molecular weight heparin (dalteparin) in Fedorak grades on mucosa of TNBS-induced colitis in rats

	n	24 h	Day 7	Day 14
Control group	5	1.33±0.54	4.33±0.81 <sup>b</sup>	3.67±2.13
150 U/kg LMWH group	4	1.00±0.00	3.33±2.08	1.67±1.15
300 U/kg LMWH group	5	0.57±0.33	2.00±1.33	0.66±0.32 <sup>d</sup>

<sup>b</sup>P<0.01 vs control group at 24 h; <sup>d</sup>P<0.01 vs control group on day 14.

**Table 2** Effect of low molecular weight heparin (dalteparin) on scores of mucosal TNF- $\alpha$  expression in rats with TNBS-induced colitis

	n	24 h	d 7	d 14
Control group	5	217±44 <sup>a</sup>	48±11	50±18
150 U/kg LMWH group	4	264±42 <sup>a</sup>	46±21	48±16
300 U/kg LMWH group	5	198±38 <sup>a,b</sup>	26±18	16±8

<sup>a</sup>P<0.001 vs control group on day 7 and 14; <sup>b</sup>P<0.05 vs 150 U/kg group at 24 h.

#### Platelet surface P-selectin expression and serum interleukin 8 production

Expression of platelet surface P-selectin was increased on d 7 and 14 in all three groups as shown in Table 3, but reached a significant level only in control group. There were no significant differences among these three groups at the three time points, 24 h, d 7 and 14. Production of serum interleukin 8 was higher at 24 h in the three groups, but significantly decreased on day 14 compared with that at 24 h in 300 U/kg LMWH treated group as shown in Table 4.

**Table 3** Effects of low molecular weight heparin (dalteparin) on platelet surface P-selectin expression (moleculars/per platelet) in rats with TNBS-induced colitis

	n	24 h	d 7	d 14
Control group	5	177.50±88.60	657.23±300.90 <sup>a</sup>	767.50±359.11 <sup>b</sup>
150 U/kg LMWH group	4	193.67±59.98	521.95±200.10	534.61±16.13
300 U/kg LMWH group	5	211.72±72.23	598.23±233.70	653.90±286.70

<sup>a</sup>P<0.05 vs control group at 24 h; <sup>b</sup>P<0.01 vs control group at 24 h.

**Table 4** Effects of low molecular weight heparin (dalteparin) on production of serum IL-8 (pg/ml) in rats with TNBS-induced colitis

	n	24 h	d 7	d 14
Control group	5	7.50±3.50	4.51±2.59	4.53±3.37
150 U/kg LMWH group	4	8.38±4.01	5.04±2.01	4.44±2.88
300 U/kg LMWH group	5	8.11±3.87	3.09±1.28	2.06±1.03 <sup>a</sup>

<sup>a</sup>P<0.05 vs 300 U/kg LMWH group at 24 h.

## DISCUSSION

In this study we observed an anti-inflammatory effect of LMWH (dalteparin) in rats with TNBS-induced colitis. This effect was demonstrated by improvement of colonic inflammation with

macroscopic and histological alterations in a dose of 300 U/kg of heparin treatment for 14 d. The result was similar to that of Fries *et al*<sup>[26]</sup>, in which they showed that heparin could prevent TNBS-induced colitis, but steroids could not. Our data showed that serum IL-8 production in 300 U/kg LMWH treated group was significantly lower on d 14 than that at 24 h, but we did not find this variation in other groups. Expression of platelet surface P-selectin in control group was significantly increased consecutively at 24 h, d 7 and 14, but expression of platelet surface P-selectin did not increase in LMWH treatment group. We also did not find differences of expression of TNF- $\alpha$  in colonic mucosa between LMWH treatment group and control group. Our results suggested that LMWH (dalteparin) in a high dose had anti-inflammatory effects. The effects were possibly related to the decrease of proinflammatory cytokine IL-8, but not related to platelet surface P-selectin.

We observed dose and time-dependent effects of LMWH in rats with TNBS-induced colitis. Three hundreds U/kg LMWH treatment group showed more histological improvement of colitis, lower TNF- $\alpha$  expression in mucosa and serum IL-8 production than 150 U/kg LMWH treated group. With a continuing LMWH treatment these effects were gradually demonstrated on d 7 and 14. Our result was slightly different from that of Dotan *et al*<sup>[18]</sup>. They showed that a single dose of 80  $\mu$ g/kg of LMWH (enoxaparin) was more optimal for amelioration of dinitrobenzene sulphonic acid- and iodoacetamide-induced colitis in rats than a dose of 200  $\mu$ g/kg and 40  $\mu$ g/kg of enoxaparin. The mechanism is not known, but may be related to optimal interactions between LMWH fragments and their receptors.

Chowers *et al*<sup>[27]</sup> found that disaccharides derived from heparin sulfate and heparin could suppress IL-8 and IL-1 $\beta$  production in intestinal epithelial cells *in vivo*. Our result was similar to that of the treatment of LMWH (dalteparin), serum IL-8 was much decreased. Salas *et al*<sup>[28]</sup> also showed that heparin pretreatment significantly attenuated leukocyte rolling, adhesion, and migration *ex vivo* but did not affect the expression of cell adhesion molecules or vascular permeability elicited by TNF- $\alpha$ . The effects of heparin involved attenuation of a CD11b dependent adherent mechanism. Nelson *et al*<sup>[29]</sup> found that *in vitro* heparin tetrasaccharides reduced binding of neutrophils to COS cells expressing P-selectin but not to COS cells expressing E-selectin.

As for the side effects of heparin in the treatment of IBD, intestinal bleeding was mentioned in several studies<sup>[30]</sup>. Our study also showed more severe intestinal bleeding in LMWH treatment group than in control group at 24 h by macroscopic and histological observations. Thus, we should be cautious of using LMWH for clinical treatment of IBD.

In conclusion, our data indicate that LMWH has an anti-inflammatory effect in TNBS-induced colitis. The mechanism is possibly related to inhibition of proinflammatory cytokine, IL-8, but not to platelet surface P-selectin production.

## REFERENCES

- 1 Musio F, Older SA, Jenkins T, Gregorie EM. Case report: cerebral venous thrombosis as a manifestation of acute ulcerative colitis. *Am J Med Sci* 1993; **305**: 28-35
- 2 Thornton M, Solomon MJ. Crohn's disease: in defense of a microvascular aetiology. *Int J Colorectal Dis* 2002; **17**: 287-297
- 3 Mutlu B, Ermeidan CM, Enc F, Fotbolcu H, Demirkol O, Bayrak F, Basaran Y. Acute myocardial infarction in a young woman with severe ulcerative colitis. *Int J Cardiol* 2002; **83**: 183-185
- 4 Srivastava AK, Khanna N, Sardana V, Gaekwad S, Prasad K, Behari M. Cerebral venous thrombosis in ulcerative colitis. *Neurol India* 2002; **50**: 215-217
- 5 Crowe A, Taffinder N, Layer GT, Irvine A, Nicholls RJ. Portal vein thrombosis in a complicated case of Crohn's disease. *Postgrad*

- Med J* 1992; **68**: 291-293
- 6 **Nguyen LT**, Laberge JM, Guttman FM, Albert D. Spontaneous deep vein thrombosis in childhood and adolescence. *J Pediatr Surg* 1986; **21**: 640-643
- 7 **Braverman D**, Bogoch A. Arterial thrombosis in ulcerative colitis. *Am J Dig Dis* 1978; **23**: 1148-1150
- 8 **Ryan FP**, Timperley WR, Preston FE, Holdsworth CD. Cerebral involvement with disseminated intravascular coagulation in intestinal disease. *J Clin Pathol* 1977; **30**: 551-555
- 9 **Mannaioni PF**, Di Bello MG, Masini E. Platelets and inflammation: role of platelet-derived growth factor, adhesion molecules and histamine. *Inflamm Res* 1997; **46**: 4-18
- 10 **Collins CE**, Cahill MR, Newland AC, Rampton DS. Platelets circulate in an activated state in inflammatory bowel disease. *Gastroenterology* 1994; **106**: 840-845
- 11 **Schaufelberger HD**, Uhr MR, McGuckin C, Logan RP, Misiewicz JJ, Gordon-Smith EC, Beglinger C. Platelets in ulcerative colitis and Crohn's disease express functional interleukin-1 and interleukin-8 receptors. *Eur J Clin Invest* 1994; **24**: 656-663
- 12 **Collins CE**, Rampton DS. Review article: platelets in inflammatory bowel disease—pathogenetic role and therapeutic implications. *Aliment Pharmacol Ther* 1997; **11**: 237-247
- 13 **Levine A**, Kenet G, Bruck R, Avni Y, Avinoach I, Aeed H, Matas Z, David M, Yayon A. Effect of heparin on tissue binding activity of fibroblast growth factor and heparin-binding epidermal growth factor in experimental colitis in rats. *Pediatr Res* 2002; **51**: 635-640
- 14 **Tyrrell DJ**, Horne AP, Holme KR, Preuss JM, Page CP. Heparin in inflammation: potential therapeutic applications beyond anticoagulation. *Adv Pharmacol* 1999; **46**: 151-208
- 15 **Cahalon L**, Lider O, Schor H, Avron A, Gilat D, HersHKoviz R, Margalit R, Eshel A, Shoseyev O, Cohen IR. Heparin disaccharides inhibit tumor necrosis factor- $\alpha$  production by macrophages and arrest immune inflammation in rodents. *Int Immunol* 1997; **9**: 1517-1522
- 16 **Vrij AA**, Jansen JM, Schoon EJ, de Bruine A, Hemker HC, Stockbrugger RW. Low molecular weight heparin treatment in steroid refractory ulcerative colitis: clinical outcome and influence on mucosal capillary thrombi. *Scand J Gastroenterol Suppl* 2001; **234**: 41-47
- 17 **Dotan I**, Hallak A, Arber N, Santo M, Alexandrowitz A, Knaani Y, HersHKoviz R, Brazowski E, Halpern Z. Low-dose low-molecular weight heparin (enoxaparin) is effective as adjuvant treatment in active ulcerative colitis: an open trial. *Dig Dis Sci* 2001; **46**: 2239-2244
- 18 **Dotan I**, HersHKoviz R, Karmeli F, Brazowski E, Peled Y, Rachmilewitz D, Halpern Z. Heparin and low-molecular-weight heparin (enoxaparin) significantly ameliorate experimental colitis in rats. *Aliment Pharmacol Ther* 2001; **15**: 1687-1697
- 19 **Torkvist L**, Thorlacius H, Sjoqvist U, Bohman L, Lapidus A, Flood L, Agren B, Raud J, Lofberg R. Low molecular weight heparin as adjuvant therapy in active ulcerative colitis. *Aliment Pharmacol Ther* 1999; **13**: 1323-1328
- 20 **Folwaczny C**, Wiebecke B, Loeschke K. Unfractionated heparin in the therapy of patients with highly active inflammatory bowel disease. *Am J Gastroenterol* 1999; **94**: 1551-1555
- 21 **Evans RC**, Wong VS, Morris AI, Rhodes JM. Treatment of corticosteroid-resistant ulcerative colitis with heparin—a report of 16 cases. *Aliment Pharmacol Ther* 1997; **11**: 1037-1040
- 22 **Michell NP**, Lalor P, Langman MJ. Heparin therapy for ulcerative colitis? Effects and mechanisms. *Eur J Gastroenterol Hepatol* 2001; **13**: 449-456
- 23 **Papa A**, Danese S, Gasbarrini A, Gasbarrini G. Review article: potential therapeutic applications and mechanisms of action of heparin in inflammatory bowel disease. *Aliment Pharmacol Ther* 2000; **14**: 1403-1409
- 24 **Morris GP**, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL. Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology* 1989; **96**: 795-803
- 25 **Fedorak RN**, Empey LR, MacArthur C, Jewell LD. Misoprostol provides a colonic mucosal protective effect during acetic acid-induced colitis in rats. *Gastroenterology* 1990; **98**: 615-625
- 26 **Fries W**, Pagiaro E, Canova E, Carraro P, Gasparini G, Pomerri F, Martin A, Carlotto C, Mazzon E, Sturniolo GC, Longo G. The effect of heparin on trinitrobenzene sulphonic acid-induced colitis in the rat. *Aliment Pharmacol Ther* 1998; **12**: 229-236
- 27 **Chowers Y**, Lider O, Schor H, Barshack I, Tal R, Ariel A, Bar-Meir S, Cohen IR, Cahalon L. Disaccharides derived from heparin or heparan sulfate regulate IL-8 and IL-1  $\beta$  secretion by intestinal epithelial cells. *Gastroenterology* 2001; **120**: 449-459
- 28 **Salas A**, Sans M, Soriano A, Reverter JC, Anderson DC, Pique JM, Panes J. Heparin attenuates TNF- $\alpha$  induced inflammatory response through a CD11b dependent mechanism. *Gut* 2000; **47**: 88-96
- 29 **Nelson RM**, Cecconi O, Roberts WG, Aruffo A, Linhardt RJ, Bevilacqua MP. Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation. *Blood* 1993; **82**: 3253-3258
- 30 **Panes J**, Esteve M, Cabre E, Hinojosa J, Andreu M, Sans M, Fernandez-Banares F, Feu F, Gassull MA, Pique JM. Comparison of heparin and steroids in the treatment of moderate and severe ulcerative colitis. *Gastroenterology* 2000; **119**: 903-908

Edited by Zhang JZ and Wang XL

# Effect of *in vitro* interferon-beta administration on hepatitis C virus in peripheral blood mononuclear cells as a predictive marker of clinical response to interferon treatment for chronic hepatitis C

Kaori Mochizuki, Tatehiro Kagawa, Shinji Takashimizu, Kazuya Kawazoe, Sei-Ichiro Kojima, Naruhiko Nagata, Atsushi Nakano, Yasuhiro Nishizaki, Koichi Shiraishi, Masaru Itakura, Norihito Watanabe, Tetsuya Mine, Shohei Matsuzaki

**Kaori Mochizuki, Tatehiro Kagawa, Shinji Takashimizu, Kazuya Kawazoe, Sei-Ichiro Kojima, Naruhiko Nagata, Atsushi Nakano, Yasuhiro Nishizaki, Koichi Shiraishi, Masaru Itakura, Norihito Watanabe, Tetsuya Mine, Shohei Matsuzaki,** Department of Internal Medicine, Division of Gastroenterology, Tokai University School of Medicine, Bohseidai, Isehara 259-1193, Japan

**Correspondence to:** Dr. Tatehiro Kagawa, Department of Internal Medicine, Division of Gastroenterology, Tokai University School of Medicine, Bohseidai, Isehara 259-1193, Japan. kagawa@is.icc.u-tokai.ac.jp

**Telephone:** +81-463-93-1121

**Received:** 2003-10-08 **Accepted:** 2003-12-30

## Abstract

**AIM:** To test whether *in vitro* incubation of peripheral blood mononuclear cells (PBMC) with interferon (IFN) could efficiently decrease hepatitis C virus-RNA (HCV-RNA) amount and to analyze whether this effect was associated with clinical response to IFN.

**METHODS:** Twenty-seven patients with histologically proven chronic hepatitis C were given intravenous administration of 6 million units (MU) IFN- $\beta$  daily for 6 weeks followed by three times weekly for 20 weeks. PBMC collected before IFN therapy were incubated with IFN- $\beta$  and HCV-RNA in PMBC was semi-quantitatively determined.

**RESULTS:** Twenty-five patients completed IFN therapy. Eight patients (32%) had sustained loss of serum HCV-RNA with normal serum ALT levels after IFN therapy (complete responders). HCV-RNA in PBMC was detected in all patients, whereas it was not detected in PBMC from healthy subjects. *In vitro* administration of IFN- $\beta$  decreased the amount of HCV-RNA in PMBC in 18 patients (72%). Eight of these patients obtained complete response. On the other hand, none of the patients whose HCV-RNA in PBMC did not decrease by IFN- $\beta$  was complete responders. Multiple logistic regression analysis revealed that the decrease of HCV-RNA amount in PBMC by IFN- $\beta$  was the only independent predictor for complete response ( $P < 0.05$ ).

**CONCLUSION:** The effect of *in vitro* IFN- $\beta$  on HCV in PBMC reflects clinical response and would be taken into account as a predictive marker of IFN therapy for chronic hepatitis C.

Mochizuki K, Kagawa T, Takashimizu S, Kawazoe K, Kojima SI, Nagata N, Nakano A, Nishizaki Y, Shiraishi K, Itakura M, Watanabe N, Mine T, Matsuzaki S. Effect of *in vitro* interferon-beta administration on hepatitis C virus in peripheral blood mononuclear cells as a predictive marker of clinical response to interferon treatment for chronic hepatitis C. *World J Gastroenterol* 2004; 10(5): 733-736

<http://www.wjgnet.com/1007-9327/10/733.asp>

## INTRODUCTION

Interferon (IFN) is effective in the treatment of chronic hepatitis C. However, the efficacy of IFN is limited and approximately 20% of patients obtain sustained virological response<sup>[1,2]</sup>. Even the combination therapy with IFN and ribavirin induces it in 30% to 40% of patients<sup>[3-5]</sup>. Because IFN is expensive and also has potentially severe adverse effects, it is important to discriminate which patient is responsive to IFN before starting therapy. The low amount of serum hepatitis C virus (HCV)-RNA, viral genotypes other than 1b and the absence of cirrhosis are associated with favorable response<sup>[1]</sup>. However, accurate prediction is still difficult before IFN treatment and more sensitive markers related to good response are required.

HCV is a hepatotropic virus, but was reported to exist in peripheral blood mononuclear cells (PBMC)<sup>[6-8]</sup>. The existence of minus strand HCV-RNA<sup>[7,9]</sup> suggests the proliferation of HCV in PBMC. Both *in vivo* and *in vitro* administration of IFN induce antiviral enzymes such as 2',5'-oligoadenylate synthetase (2-5AS) in PBMC as well as in liver<sup>[10,11]</sup>. We hypothesized that *in vitro* administration of IFN might exert antiviral effect on HCV proliferating in PBMC. We tested whether *in vitro* incubation of PBMC with IFN could efficiently decrease HCV-RNA amount and also analyzed whether this effect was associated with clinical response to IFN.

## MATERIALS AND METHODS

### Patients and study design

Twenty-seven patients with chronic hepatitis C were enrolled into this study. Criteria for enrollment were elevation of serum ALT levels, positivity for anti-HCV antibody and serum HCV-RNA, presence of histologically proven chronic hepatitis, and absence of other chronic liver diseases such as autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, Wilson's disease. Liver histology was obtained from all patients before IFN treatment and evaluated for staging of fibrosis and grade of activity according to the METAVIR scoring system<sup>[12,13]</sup>. They were given intravenous administration of 6 million units (MU) IFN- $\beta$  (Feron, Toray Pharmaceuticals, Tokyo, Japan) daily for 6 weeks followed by three times weekly for 20 weeks. Informed consent was obtained from all patients.

The effect of IFN was defined as complete response (CR): sustained loss of serum HCV-RNA and normal ALT levels, partial response (PR): disappearance of serum HCV-RNA at the end of treatment and reappearance after treatment, and no response (NR): positive serum HCV-RNA at the end of treatment.

Serum viral load was defined by multicyclic reverse transcription (RT)-polymerase chain reaction (PCR) method<sup>[14,15]</sup> and patients were categorized into 2 groups, namely high:  $\geq 10^8$  copies/mL, and low:  $< 10^8$  copies/mL. HCV genotyping was performed with type-specific primers<sup>[16]</sup>.

### Effect of IFN- $\beta$ on PBMC

Twenty ml of peripheral blood was drawn before IFN

treatment. PBMC were separated by gradient centrifugation and washed with phosphate-buffered saline (PBS) seven times. After this procedure the supernatants were negative for HCV-RNA even after two-step PCR as described below. PBMC were suspended in RPMI-1640 medium supplemented with fetal bovine serum (IWAKI, Tokyo, Japan) at a concentration of  $2 \times 10^6$  cells/mL, plated on a 24-well plate (IWAKI), and cultured at 37 °C in a humidified atmosphere with 50 mL/L CO<sub>2</sub> in the absence or presence of IFN- $\beta$  at a concentration of 100 IU/L. After incubation for 72 h PBMC were washed five times with PBS followed by RNA extraction with guanidium thiocyanate. Total RNA was reversely transcribed into cDNA in a 40  $\mu$ L reaction mixture containing 100 pmoles antisense primer (5' -AACACTACTCGGCTAGCAGT-3') and 200 units of SuperScript II reverse transcriptase (Invitrogen Japan, Tokyo, Japan). The amplification of cDNA was performed for 35 cycles in four temperature steps (at 94 °C for 2 min, 37 °C for 2 min, 55 °C for 2 min and 72 °C for 3 min) in a 100  $\mu$ L reaction mixture containing the template, 1 unit of Taq polymerase (Perkin Elmer, Norwalk, CT), 20 pmol of each outer primer, 2.5 nmol of each deoxyribonucleotide triphosphates, 10 mmol/L Tris HCl, pH 8.3, 50 mmol/L KCl and 0.15 mmol/L MgCl<sub>2</sub>. After the first PCR, 10  $\mu$ L of the PCR product was subjected to a second PCR amplification using the inner primer pair under the same conditions as described for the first PCR. Primers were designed from the sequence of 5' non-coding region, an outer primer pair of 242-base span: sense (5' -ACTCCACCATAGATCATCCC-3') and antisense (5' -AACACTACTCGGCTAGCAGT-3') and an inner primer pair of 145-base span: sense (5' -TTCACGCAG-AAAAGCGTCTAG-3') and antisense (5' -GTTGATCCAA-GAAAGGACCC-3'). PCR products were analyzed by gel-electrophoresis. The amount of HCV-RNA in PBMC was determined semi-quantitatively as 2+: positive after first PCR; 1+: negative after first PCR and positive after second PCR; and -: negative after first and second PCR. PBMC from 5 healthy subjects were also examined for the presence of HCV-RNA.

### Statistical analysis

Categorical variables were analyzed by the chi-square test. Continuous numeric variables were examined by the Student's *t*-test (two-tail). Multiple logistic regression analysis was preformed by SPSS for Macintosh to identify independent predictors for CR.

## RESULTS

### Response to IFN treatment (Table 1)

Twenty-five of 27 patients completed IFN treatment. One discontinued IFN because of retinal hemorrhage, the other could not visit our hospital due to moving. All patients had fever, general fatigue or myalgia. However, no severe adverse effects were seen. Eight patients (32%) maintained normal serum ALT levels with negative serum HCV-RNA after IFN treatment, resulting in CR. Ten patients (40%) were negative for HCV-RNA at the end of treatment but relapsed after the discontinuation of IFN (PR). The other 7 patients (28%) were still positive for HCV-RNA at the end of IFN treatment (NR). The distribution of gender, age and serum ALT levels was not significantly different among three groups. Neither grade of activity nor staging of fibrosis in liver histology was different. Low serum viral load and genotypes other than 1b were associated with favorable response ( $P < 0.05$ ).

### HCV-RNA in PBMC (Table 2)

We analyzed the presence of HCV-RNA in PBMC by two-step RT-PCR. All patients were positive for HCV-RNA in PBMC, whereas none of the healthy subjects was positive.

Three patients (12%) revealed 1+; 2 with low serum viral load and 1 with high viral load. The other 22 patients (88%) resulted in 2+. The amount of HCV-RNA in PBMC before the addition of IFN- $\beta$  was not related to clinical response to IFN.

**Table 1** Demographic characteristics and clinical response

Variable	NR <sup>a</sup> (n=7)	PR (n=10)	CR (n=8)	P value
Sex (F:M)	4:3	8:2	6:2	NS
Age (mean $\pm$ SD)	57.2 $\pm$ 6.7	51.4 $\pm$ 7.2	44.4 $\pm$ 15.7	NS
Serum ALT levels (mean $\pm$ SD)	68.5 $\pm$ 39.5	95.4 $\pm$ 67.9	90.9 $\pm$ 40.1	NS
Liver histology				
Activity index				
A1	4	3	1	NS
A2	2	6	7	
A3	1	1	0	
Fibrosis index				
F1	3	5	5	NS
F2	3	3	2	
F3	1	2	1	
Serum viral load <sup>b</sup>				
Low	1	6	6	<0.05
High	6	4	2	
Genotype				
1b	6	7	2	<0.05
Others	1	3	6	

<sup>a</sup>Complete response (CR): sustained loss of serum HCV-RNA and normal ALT levels, partial response (PR): disappearance of serum HCV-RNA at the end of treatment and reappearance after treatment, and no response (NR): positive serum HCV-RNA at the end of treatment. <sup>b</sup>high:  $\geq 10^8$  copies/mL, low:  $< 10^8$  copies/mL.

**Table 2** Viral load in PBMC and clinical response

Variable	NR <sup>a</sup> (n=7)	PR (n=10)	CR (n=8)	P value
Viral load <sup>b</sup> (pretreatment)				
-	0	0	0	NS
1+	0	2	1	
2+	7	8	7	
Decrease of HCV-RNA by IFN- $\beta$ <sup>c</sup>				
Yes	1	9	8	<0.05
No	6	1	0	

<sup>a</sup>Complete response (CR): sustained loss of serum HCV-RNA and normal ALT levels, partial response (PR): disappearance of serum HCV-RNA at the end of treatment and reappearance after treatment, and no response (NR): positive serum HCV-RNA at the end of treatment. <sup>b</sup>The amount of HCV-RNA in PBMC was determined semi-quantitatively as follows; 2+: positive after first PCR, 1+: negative after first PCR and positive after second PCR, and -: negative after first and second PCR. <sup>c</sup>HCV-RNA amount in PBMC was semi-quantitatively determined before and after incubation with IFN- $\beta$  (100 IU/L).

We studied the effect of *in vitro* administration of IFN- $\beta$  on HCV in PBMC. We used 100 IU/L as a concentration of IFN- $\beta$  because preliminary experiments revealed that 10 IU/L of IFN- $\beta$  was insufficient to decrease HCV-RNA in PBMC and that 1 000 IU/L of IFN- $\beta$  did not have more effects than 100 IU/L of IFN- $\beta$  (data not shown). After incubation with IFN- $\beta$  the HCV-RNA amount in PBMC decreased in 18 patients (72%), 7 patients: 2+ to -; 8 patients: 2+ to 1+; and 3 patients: 1+ to -. Whereas no decrease was observed in the other 7 patients (28%). Incubation without IFN- $\beta$  did not lead

to decrease in the amount of HCV-RNA (data not shown), suggesting that this decrease was attributable to the antiviral effect of IFN- $\beta$ .

We analyzed the relationship between the effect of IFN- $\beta$  on HCV in PBMC and clinical response. Patients with HCV-RNA decrease were significantly associated with better clinical response, 8 (44%) and 9 patients (50%) obtained CR and PR, respectively. On the other hand, 6 of 7 patients (86%) whose HCV-RNA amount did not decrease by IFN resulted in NR. Multiple logistic regression analysis revealed that the decrease in HCV-RNA amount in PBMC was the only independent predictor for CR ( $P < 0.05$ ).

## DISCUSSION

In our study 8 patients (32%) obtained CR. IFN- $\beta$  therapy was as effective as IFN- $\alpha$  in the treatment of chronic hepatitis C as already reported<sup>[17-19]</sup>. In one patient (4%) IFN treatment was discontinued due to retinal hemorrhage, which was the only major adverse effect observed in this study. Retinopathy was one of the common adverse effects of IFN<sup>[20,21]</sup>. Low serum viral load and viral genotypes other than 1b were associated with good clinical response in consistent with other studies using IFN- $\alpha$ <sup>[22-25]</sup>.

We could detect HCV-RNA in PBMC before IFN therapy in all cases. HCV-RNA was not found in PBMC from healthy subjects, suggesting that the technique we used was specific for HCV. Several studies demonstrated the presence of HCV-RNA in PBMC<sup>[26-30]</sup>, although the positive rate was variable from 25% to 100%. The *in vitro* administration of IFN- $\beta$  decreased HCV-RNA in PBMC in 72% patients. Because incubation with medium alone did not affect HCV-RNA amount, this decrease would be attributable to IFN's antiviral effect. In fact Pawlotsky *et al* reported that 2-5AS activity in PBMC from patients with chronic hepatitis C was augmented by incubation with IFN<sup>[10]</sup>. Furthermore a preliminary experiment showed that *in vitro* addition of IFN at a concentration of 1 000 IU/mL reduced the HCV-RNA amount in PBMC<sup>[30]</sup>. In our study 7 patients (28%) maintained the same amount of HCV-RNA in PBMC even after incubation with IFN. It should be noted that none of these patients was complete responders. The reason why IFN could not decrease HCV-RNA in these patients is unclear. One possibility is that IFN could not induce antiviral enzymes such as 2-5AS. In this case the breakdown of IFN-induced antiviral system might be responsible. A more likely explanation is that HCV strains in these patients were resistant to antiviral enzymes. Some strains such as those with mutations in NS5A region<sup>[31]</sup> are refractory to IFN.

Clinical outcome was significantly different between those with decreased HCV-RNA amount in PBMC and those without. Multivariate analysis demonstrated the decrease of HCV-RNA amount in PBMC by IFN as the only independent predictive factor for CR. These data suggest that the effect of *in vitro* IFN- $\beta$  on HCV-RNA in PBMC reflects clinical response and would be taken into account as a predictive marker of IFN therapy for chronic hepatitis C.

## REFERENCES

- 1 **Hoofnagle JH**, di Bisceglie AM. The treatment of chronic viral hepatitis. *N Engl J Med* 1997; **336**: 347-356
- 2 **Poynard T**, Bedossa P, Chevallier M, Mathurin P, Lemonnier C, Trepo C, Couzigou P, Payen JL, Sajus M, Costa JM. A comparison of three interferon alfa-2b regimens for the long-term treatment of chronic non-A, non-B hepatitis. Multicenter Study Group. *N Engl J Med* 1995; **332**: 1457-1462
- 3 **McHutchison JG**, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1485-1492
- 4 **Davis GL**, Esteban-Mur R, Rustgi V, Hoefs J, Gordon SC, Trepo C, Shiffman ML, Zeuzem S, Craxi A, Ling MH, Albrecht J. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1493-1499
- 5 **Di Bisceglie AM**, McHutchison J, Rice CM. New therapeutic strategies for hepatitis C. *Hepatology* 2002; **35**: 224-231
- 6 **Chang TT**, Young KC, Yang YJ, Lei HY, Wu HL. Hepatitis C virus RNA in peripheral blood mononuclear cells: comparing acute and chronic hepatitis C virus infection. *Hepatology* 1996; **23**: 977-981
- 7 **Lerat H**, Berby F, Traubaud MA, Vidalin O, Major M, Trepo C, Inchauspe G. Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. *J Clin Invest* 1996; **97**: 845-851
- 8 **Rodriguez-Inigo E**, Casqueiro M, Navas S, Bartolome J, Pardo M, Carreno V. Fluorescent "in situ" hybridization of hepatitis C virus RNA in peripheral blood mononuclear cells from patients with chronic hepatitis C. *J Med Virol* 2000; **60**: 269-274
- 9 **Willems M**, Peerlinck K, Moshage H, Deleu I, Van den Eynde C, Vermeylen J, Yap SH. Hepatitis C virus-RNAs in plasma and in peripheral blood mononuclear cells of hemophiliacs with chronic hepatitis C: evidence for viral replication in peripheral blood mononuclear cells. *J Med Virol* 1994; **42**: 272-278
- 10 **Pawlotsky JM**, Hovanessian A, Roudot-Thoraval F, Lebon P, Robert N, Bouvier M, Babany G, Duval J, Dhumeaux D. Activity of the interferon-induced 2' ,5' -oligoadenylate synthetase in patients with chronic hepatitis C. *J Interferon Cytokine Res* 1995; **15**: 857-862
- 11 **Grander D**, Sangfelt O, Skoog L, Hansson J. *In vivo* induction of the interferon-stimulated protein 2' 5' -oligoadenylate synthetase in tumor and peripheral blood cells during IFN- $\alpha$  treatment of metastatic melanoma. *J Interferon Cytokine Res* 1998; **18**: 691-695
- 12 **The French METAVIR Cooperative Study Group**. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. *Hepatology* 1994; **20**: 15-20
- 13 **Bedossa P**, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; **24**: 289-293
- 14 **Ishiyama N**, Katayama K, Ishimi N, Takahashi S, Igarashi H, Nakajima H, Andoh T, Saito S, Aoyagi T. Measurement of copy-number of hepatitis C virus by multicyclic RT-PCR. *Nippon Shokakibyo Gakkai Zasshi* 1992; **89**: 1396
- 15 **Ishiyama N**, Katayama K, Ishimi N, Takahashi S, Igarashi H, Nakajima H, Saito S, Aoyagi T, Andoh T, Oya A. Quantitative detection of hepatitis C virus RNA by multicyclic RT-PCR. *Int Hepatol Com* 1993; **1**: 72-79
- 16 **Okamoto H**, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, Tanaka T, Sato K, Tsuda F, Miyakawa Y. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. *J Gen Virol* 1992; **73**(Pt 3): 673-679
- 17 **Asahina Y**, Izumi N, Uchiyama M, Noguchi O, Tsuchiya K, Hamano K, Kanazawa N, Itakura J, Miyake S, Sakai T. A potent antiviral effect on hepatitis C viral dynamics in serum and peripheral blood mononuclear cells during combination therapy with high-dose daily interferon alfa plus ribavirin and intravenous twice-daily treatment with interferon beta. *Hepatology* 2001; **34**: 377-384
- 18 **Furusyo N**, Hayashi J, Ohmiya M, Sawayama Y, Kawakami Y, Ariyama I, Kinukawa N, Kashiwagi S. Differences between interferon-alpha and -beta treatment for patients with chronic hepatitis C virus infection. *Dig Dis Sci* 1999; **44**: 608-617
- 19 **Kobayashi Y**, Watanabe S, Konishi M, Yokoi M, Kakehashi R, Kaito M, Kondo M, Hayashi Y, Jomori T, Suzuki S. Quantitation and typing of serum hepatitis C virus RNA in patients with chronic hepatitis C treated with interferon-beta. *Hepatology* 1993; **18**: 1319-1325
- 20 **Hayasaka S**, Nagaki Y, Matsumoto M, Sato S. Interferon associated retinopathy. *Br J Ophthalmol* 1998; **82**: 323-325
- 21 **Tsolakos A**, Zalatio N. Hepatitis C: a review of diagnosis,

- management, and ocular complications from treatment. *Optometry* 2003; **74**: 517-523
- 22 **Chemello L**, Bonetti P, Cavalletto L, Talato F, Donadon V, Casarin P, Belussi F, Frezza M, Noventa F, Pontisso P. Randomized trial comparing three different regimens of alpha-2a-interferon in chronic hepatitis C. The TriVeneto Viral Hepatitis Group. *Hepatology* 1995; **22**: 700-706
- 23 **Tsubota A**, Chayama K, Ikeda K, Yasuji A, Koida I, Saitoh S, Hashimoto M, Iwasaki S, Kobayashi M, Hiromitsu K. Factors predictive of response to interferon-alpha therapy in hepatitis C virus infection. *Hepatology* 1994; **19**: 1088-1094
- 24 **Martinot-Peignoux M**, Marcellin P, Pouteau M, Castelnau C, Boyer N, Poliquin M, Degott C, Descombes I, Le Breton V, Milotova V. Pretreatment serum hepatitis C virus RNA levels and hepatitis C virus genotype are the main and independent prognostic factors of sustained response to interferon alfa therapy in chronic hepatitis C. *Hepatology* 1995; **22**: 1050-1056
- 25 **Nousbaum JB**, Pol S, Nalpas B, Landais P, Berthelot P, Brechot C. Hepatitis C virus type 1b (II) infection in France and Italy. Collaborative Study Group. *Ann Intern Med* 1995; **122**: 161-168
- 26 **Trimoulet P**, Bernard PH, de Ledinghen V, Oui B, Chene G, Saint-Marc Girardin MF, Dantin S, Couzigou P, Fleury H. Quantitation of hepatitis C virus RNA in plasma and peripheral blood mononuclear cells of patients with chronic hepatitis treated with interferon-alpha. *Dig Dis Sci* 2000; **45**: 175-181
- 27 **Kao JH**, Chen PJ, Lai MY, Wang TH, Chen DS. Positive and negative strand of hepatitis C virus RNA sequences in peripheral blood mononuclear cells in patients with chronic hepatitis C: no correlation with viral genotypes 1b, 2a, and 2b. *J Med Virol* 1997; **52**: 270-274
- 28 **Ounanian A**, Gueddah N, Rolachon A, Thelu MA, Zarski JP, Seigneurin JM. Hepatitis C virus RNA in plasma and blood mononuclear cells in patients with chronic hepatitis C treated with alpha-interferon. *J Med Virol* 1995; **45**: 141-145
- 29 **Taliani G**, Badolato C, Lecce R, Poliandri G, Bozza A, Duca F, Pasquazzi C, Clementi C, Furlan C, De Bac C. Hepatitis C virus RNA in peripheral blood mononuclear cells: relation with response to interferon treatment. *J Med Virol* 1995; **47**: 16-22
- 30 **Martin J**, Navas S, Fernandez M, Rico M, Pardo M, Quiroga JA, Zahm F, Carreno V. *In vitro* effect of amantadine and interferon alpha-2a on hepatitis C virus markers in cultured peripheral blood mononuclear cells from hepatitis C virus-infected patients. *Antiviral Res* 1999; **42**: 59-70
- 31 **Enomoto N**, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; **334**: 77-81

Edited by Wang XL Proofread by Zhu LH



# Factors related to lymph node metastasis and surgical strategy used to treat early gastric carcinoma

Dong Yi Kim, Jae Kyoong Joo, Seong Yeob Ryu, Young Jin Kim, Shin Kon Kim

**Dong Yi Kim, Jae Kyoong Joo, Seong Yeob Ryu, Young Jin Kim, Shin Kon Kim**, Division of Gastroenterologic Surgery, Department of Surgery, Chonnam National University Medical School, Gwangju, Korea

**Correspondence to:** Dr. Dong Yi Kim, Division of Gastroenterologic Surgery, Department of Surgery, Chonnam National University Hospital, 8, Hakdong, Dongku, Gwangju, 501-757, Korea. dockim@chonnam.ac.kr

**Telephone:** +82-62-220-6450 **Fax:** +82-62-227-1635

**Received:** 2003-10-29 **Accepted:** 2003-12-16

## Abstract

**AIM:** The prognosis of early gastric carcinoma (EGC) is generally excellent after surgery. The presence or absence of lymph node metastasis in EGC is an important prognostic factor. The survival and recurrence rates of node-negative EGC are much better than those of node-positive EGC. This study examined the factors related to lymph node metastasis in EGC to determine the appropriate treatment for EGC.

**METHODS:** We investigated 748 patients with EGC who underwent surgery between January 1985 and December 1999 at the Division of Gastroenterologic Surgery, Department of Surgery, Chonnam National University Hospital. Several clinicopathologic factors were investigated to analyze their relationship to lymph node metastasis: age, sex, tumor location, tumor size, gross type, histologic type, depth of invasion, extent of lymph node dissection, type of operation, and DNA ploidy.

**RESULTS:** Lymph node metastases were found in 75 patients (10.0%). Univariate analysis showed that male sex, tumor size larger than 2.0 cm, submucosal invasion of tumor, histologic differentiation, and DNA ploidy pattern were risk factors for regional lymph node metastasis in EGC patients. However, a multivariate analysis showed that three risk factors were associated with lymph node metastasis: large tumor size, undifferentiated histologic type and submucosal invasion. No statistical relationship was found for age, sex, tumor location, gross type, or DNA ploidy in multivariate analysis. The 5-year survival rate was 94.2% for those without lymph node metastasis and 87.3% for those with lymph node metastasis, and the difference was significant ( $P < 0.05$ ).

**CONCLUSION:** In patients with EGC, the survival rate of patients with positive lymph nodes is significantly worse than that of patients with no lymph node metastasis. Therefore, a standard D2 lymphadenectomy should be performed in patients at high risk of lymph node metastasis: large tumor size, undifferentiated histologic type and submucosal invasion.

Kim DY, Joo JK, Ryu SY, Kim YJ, Kim SK. Factors related to lymph node metastasis and surgical strategy used to treat early gastric carcinoma. *World J Gastroenterol* 2004; 10(5): 737-740  
<http://www.wjgnet.com/1007-9327/10/737.asp>

## INTRODUCTION

Early gastric carcinoma (EGC) is defined as a lesion confined to the mucosa or submucosa of the stomach with or without lymph node metastasis<sup>[1]</sup>. The incidence of lymph node metastasis differs in mucosal and submucosal gastric carcinomas. When invasion extends to the submucosal layer, the incidence of lymph node metastasis increases<sup>[2]</sup> and submucosal gastric carcinoma is reported to have a poorer prognosis than mucosal gastric carcinoma<sup>[3]</sup>.

We analyzed data from 673 patients with node-negative EGC, and 75 patients with node-positive EGC who had been surgically treated, to evaluate the clinicopathologic factors related to lymph node metastasis in EGC and to determine the appropriate treatment criteria for patients with EGC.

## MATERIALS AND METHODS

Between 1985 and 1999, 748 Korean patients with EGC underwent surgery at the Division of Gastroenterologic Surgery, Department of Surgery, Chonnam National University Hospital. Of these, 75 patients (10.0%) were found to have lymph node metastasis.

Medical records were reviewed, and data were abstracted for the following factors: clinical findings, tumor size, tumor location, gross appearance, histologic grade, lymph node involvement, operation type, DNA ploidy pattern, and 5-year survival rate.

The clinical features of the 673 patients with node-negative EGC, and the 75 patients with node-positive EGC were compared. Curative resections were defined as histologic or relative curative resections according to the criteria of the Japanese Research Society for Gastric Cancer<sup>[4]</sup>. The data were analyzed statistically using a chi-square test and an unpaired Student's *t*-test. Overall survival rates were calculated using the Kaplan-Meier method. Multivariate analysis was performed using the Cox proportional hazards model with the program SPSS 11.0 to test the variables associated with lymph node metastasis in EGC. A *P* value  $< 0.05$  was considered statistically significant.

## RESULTS

Of the 2 767 patients with gastric carcinoma who underwent surgery in our hospital over the 15-year period, 748 patients (27.0%) were diagnosed as EGC. The incidence of node-positive EGC was 10.0% (75/748) in this study group.

Table 1 describes the clinicopathologic findings in the 673 patients with node-negative EGC, and the 75 patients with node-positive EGC. There was no statistically significant difference between the ages of patients with and without lymph node metastasis. Of the 75 patients with lymph node metastasis, 41 (54.7%) were males and 34 (45.3%) were females. There were 453 males (67.3%) and 220 females (32.7%) in the group of 673 patients with node-negative EGC. There were more males than females in each group and the gender ratio was statistically significant ( $P < 0.05$ ). The mean tumor size of patients with node-positive EGC (3.1 cm) was larger than that of patients with node-negative EGC (2.2 cm), and the difference was statistically significant ( $P < 0.01$ ). Lymph node metastasis was

present in only 10 patients (13.3%) with a tumor <2.0 cm in size. Of these 75 patients with node-positive EGC, 65 patients (86.7%) were found to have a tumor  $\geq 2.0$  cm in size ( $P < 0.01$ ). Most gastric carcinomas were located in the lower portion of the stomach, in both node-negative (445 cases, 66.1%) and node-positive EGC patients (55 cases, 73.3%), but the differences in location were not significant. There was no correlation between lymph node metastasis and macroscopic appearance of the tumor. Based on the degree of anaplasia, 10 (13.3%) patients with node-positive EGC had well-differentiated, 14 (18.7%) had moderately differentiated, 45 (60.0%) had poorly differentiated, and 3 (4.0%) had mucinous adenocarcinomas ( $P < 0.01$ ). The submucosal invasion was found more frequently in patients with node-positive EGC (81.3%) than in those with node-negative EGC (46.1%,  $P < 0.01$ ). Distant metastasis was found in one patient with node-negative EGC. There was no significant difference between node-negative and node-positive EGC patients in the operative type. The curative resection rate for patients with node-negative EGC was similar to that for patients with node-positive EGC (99.7% vs 97.3%).

**Table 1** Clinicopathologic findings in patients with early gastric carcinoma with and without lymph node metastasis

	Node-negative <i>n</i> =673, (%)	Node-positive <i>n</i> =75, (%)	<i>P</i> value
Age (mean, yr)	55.7 $\pm$ 10.7	57.9 $\pm$ 10.7	NS
Gender			<0.05
Male	453 (67.3)	41 (54.7)	
Female	220 (32.7)	34 (45.3)	
Age (yr)			NS
$\leq 40$	71 (10.5)	5 (6.7)	
> 40	602 (89.5)	70 (93.3)	
Tumor size (mean, cm)	2.2 $\pm$ 1.45	3.1 $\pm$ 1.89	<0.01
< 2	434 (64.5)	10 (13.3)	<0.01
2-3	141 (21.0)	24 (32.0)	
> 3	98 (14.5)	41 (54.7)	
Tumor location			NS
Upper	28 (4.2)	2 (2.7)	
Middle	200 (29.7)	18 (24.0)	
Lower	445 (66.1)	55 (73.3)	
Macroscopic appearance			NS
Protruded	208 (30.9)	21 (28.0)	
Depressed	417 (62.0)	50 (66.7)	
Mixed	48 (7.1)	4 (5.3)	
Stage			<0.01
Ia	672 (99.9)		
Ib		65 (86.7)	
II		6 (8.0)	
IV	1 (0.1)	4 (5.3)	
Histologic type			<0.01
Differentiated	348 (51.7)	24 (32.0)	
Undifferentiated	325 (48.3)	51 (68.0)	
Depth of invasion			<0.01
Mucosa	363 (53.9)	14 (18.7)	
Submucosa	310 (46.1)	61 (81.3)	
Operative type			NS
Total gastrectomy	62 (9.2)	8 (10.7)	
Proximal gastrectomy	4 (0.6)		
Distal gastrectomy	583 (86.8)	65 (86.7)	
Others	24 (3.4)	2 (2.7)	
Lymph node dissection			<0.01
D1	144 (21.4)	8 (10.7)	
D2	484 (71.9)	50 (66.7)	
$\geq$ D3	45 (6.7)	17 (22.7)	
Curability			NS
Curative	671 (99.7)	73 (97.3)	
Non-curative	2 (0.3)	2 (2.7)	

NS, not significant.

Univariate analysis showed that male sex, tumor size, depth of invasion, undifferentiated type, and aneuploid pattern were significant factors associated with lymph node metastasis in EGC (Tables 1 and 2).

**Table 2** Lymph node metastasis according to DNA ploidy pattern (*n*=238)

DNA ploidy	Node-negative (%)	Node-positive (%)	<i>P</i> value
Aneuploid	80 (38.5)	17 (56.7)	<0.05
Diploid	128 (61.5)	13 (43.3)	

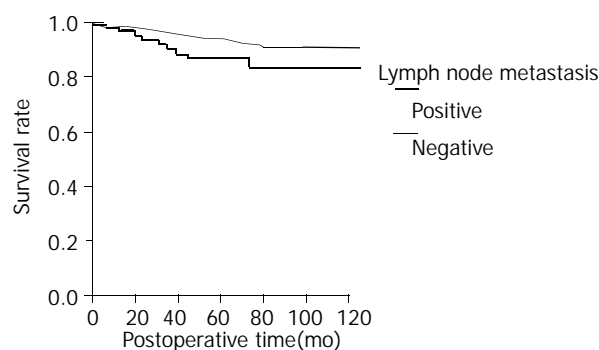
**Table 3** Logistic regression analysis for variables associated with lymph node metastasis in early gastric carcinoma

Variables	Risk ratio	95% CI	<i>P</i> value
Age	1.040	0.996-1.021	0.50
Gender	1.022	0.884-1.121	0.183
Tumor size	1.342	1.134-1.642	0.003
Gross appearance	1.124	0.987-1.342	0.384
Histologic type	0.948	0.737-1.221	0.009
Depth of invasion	1.241	0.949-1.369	<0.001
Tumor location	0.963	0.822-1.522	0.09
DNA ploidy	0.876	0.744-1.675	0.06

CI, confidence interval.

All of the factors listed in Tables 1 and 2 were examined using a logistic regression analysis. The independent risk factors for lymph node metastasis were larger tumor size, undifferentiated type, and submucosal invasion (Table 3).

The postoperative survival rate of the patients without lymph node metastasis was compared with that obtained from those with lymph node metastasis. The 5-year survival rates were 94.2% for those without lymph node metastasis and 87.3% for those with lymph node metastasis, and the difference was significant ( $P < 0.05$ , Figure 1).



**Figure 1** Survival curves according to lymph node metastasis. The early gastric carcinoma patients without lymph node metastasis showed a better survival rate than early gastric carcinoma patients with lymph node metastasis (94.2% vs 87.3%,  $P < 0.05$ ).

## DISCUSSION

Early gastric carcinoma (EGC) has been defined as gastric carcinoma in which invasion is confined to the mucosa or submucosa, regardless of lymph node metastasis<sup>[1]</sup>. The incidence of EGC has increased due to the advances in technical developments regarding both radiological modalities and endoscopy<sup>[5]</sup>. In our department, 27.0% of the patients had EGC, a rate similar to the 20-40% range previously reported<sup>[6]</sup>. The prognosis of EGC after curative resection was favorable,

with a 5-year survival rate exceeding 90%<sup>[6-8]</sup>. Moreover, the survival and recurrence rates of node-negative EGC were much better than those of node-positive EGC<sup>[7,8]</sup>.

The reported incidence of lymph node metastasis in EGC was 2-4% in mucosal carcinoma<sup>[9-11]</sup>, but increased to 15-25% in submucosal carcinoma<sup>[12]</sup>. Here, we found a 3.7% incidence of lymph node metastasis in mucosal carcinoma and a 16.4% incidence in submucosal carcinoma. The incidence of lymph node metastasis was similar to those in mucosal carcinoma reported by Yamao *et al*<sup>[9]</sup> and Tsujitani *et al*<sup>[10]</sup>, and lower in submucosal carcinoma reported by other investigators<sup>[13,14]</sup>. Some investigators have reported that submucosal invasion is one of the predictive risk factors for lymph node metastasis in EGC patients<sup>[8,15,16]</sup>. Shimada *et al* reported that the number of metastatic lymph nodes occurring in patients with gastric carcinoma was correlated with the survival rate<sup>[6]</sup>. EGC patients with lymph node metastasis had a lower survival rate than patients without lymph node metastasis. Of note, they suggested that the involvement of three or more lymph nodes could predict a poor prognosis in submucosal gastric carcinoma. Seto *et al*<sup>[17]</sup> reported a 5-year survival of 74% in patients with more than 4 positive lymph nodes. Folli *et al*<sup>[11]</sup> also reported a lower 5-year survival, especially for patients who presented more than 3 metastatic lymph nodes.

The presence of lymph node metastasis in EGC worsened the prognosis, as reported by some investigators<sup>[8,13]</sup> and as observed in this study (87.3% vs 94.2%). Nio *et al*<sup>[18]</sup> reported that the 5-year survival of EGC was 93% for N1 patients and 68.4% for N2 patients. Miwa *et al*<sup>[15]</sup> observed similar results. When EGC was subdivided into mucosal and submucosal carcinomas, the survival rate for mucosal carcinoma was significantly better than that for submucosal carcinoma<sup>[19]</sup> because the former had a lower incidence of lymph node metastasis. Nevertheless, submucosal invasion could not always predict a poor prognosis, the survival rates in patients with submucosal carcinoma were the same as those in patients with mucosal carcinoma<sup>[20]</sup>. In our study, the incidence of lymph node metastasis from submucosal carcinoma (16.4%) was significantly higher than that from mucosal carcinoma (3.7%). However, the 5-year survival rate for patients with submucosal carcinoma (88.6%) did not differ from that for patients with mucosal carcinoma (95.2%), because the curative resection rate was high for both submucosal (97.3%) and mucosal (99.7%) carcinomas. We performed gastrectomy with D2 lymphadenectomy for most patients with submucosal gastric carcinoma. Furthermore, some investigators<sup>[12,21]</sup> have recommended that a standard D2 lymphadenectomy is essential, even in cases of mucosal carcinoma.

Lymphadenectomy, a prognostic factor that can be influenced by the surgeon, improves the survival rate in gastric carcinoma, although there has been no extensive prospective randomized trial. Viste *et al*<sup>[22]</sup> reported that the survival of patients who underwent extensive lymph node dissection was higher than that of patients without dissection. Furthermore, lymph node recurrence has been attributed to inadequate lymph node dissection. We found that in patients with submucosal gastric carcinoma, the survival rate with positive lymph nodes was significantly poorer than that with no lymph node metastasis (87.3% vs 94.2%,  $P < 0.05$ ). Therefore, we recommend gastrectomy with D2 lymphadenectomy as the appropriate operative procedure for patients with submucosal carcinoma of the stomach.

There have been several attempts to identify risk factors predicting lymph node metastasis. Maehara *et al*<sup>[6]</sup> found that the risk factors for lymph node metastasis in EGC patients were large tumor, lymphatic involvement, and submucosal invasion. Yamao *et al*<sup>[9]</sup> also reported that lymphatic invasion, histologic type, and large tumor size were independent risk

factors for lymph node metastasis in patients with intramucosal EGC. Abe *et al*<sup>[23]</sup> reported that submucosal invasion, female sex, large tumor size, and lymphatic vessel involvement were significantly and independently related to the presence of lymph node metastasis in depressed EGC. Baba *et al*<sup>[24]</sup> reported that there was no metastasis in lesions less than 1 cm in diameter, but the incidence of positive nodes increased with the size of the primary lesion. Wu *et al*<sup>[25]</sup> reported that poor differentiation, submucosal invasion and large tumor size were independent risk factors for lymph node metastasis in early gastric cancer. Macroscopic classification was not correlated with lymph node metastasis. Sasaki *et al*<sup>[26]</sup> reported that DNA aneuploidy was a useful indicator of lymph node metastasis in EGC patients. We studied the DNA ploidy pattern in 238 patients with EGC and found that there was a correlation between lymph node metastasis and DNA ploidy pattern in univariate analysis. But we found there was no correlation between lymph node metastasis and DNA ploidy pattern in multivariate analysis (Table 3). In our study, the univariate analysis showed that lymph node metastasis in EGC patients was associated with male sex, large tumor size, submucosal invasion, and undifferentiated histologic grade, while the multivariate analysis showed that metastasis was associated with large tumor size, undifferentiated type and submucosal invasion (Table 3).

In conclusion, this study suggests that tumor size, depth of tumor invasion and undifferentiated histologic grade are risk factors for lymph node metastasis in EGC. Therefore, standard D2 lymphadenectomy should be performed in patients with these high-risk factors.

## REFERENCES

- 1 **Kajitani T.** The general rules for the gastric cancer study in surgery and pathology. Part I. Clinical classification. *Jpn J Surg* 1981; **11**: 127-139
- 2 **Inoue K,** Tobe T, Kan N, Nio Y, Sakai M, Takekuchi E, Sugiyama T. Problems in the definition and treatment of early gastric cancer. *Br J Surg* 1991; **78**: 818-821
- 3 **Hioki K,** Nakane Y, Yamamoto M. Surgical strategy for early gastric cancer. *Br J Surg* 1990; **77**: 1330-1334
- 4 **Japanese Research Society for Gastric Cancer.** The general rules for the gastric cancer study in surgery and pathology. 12<sup>th</sup> ed. Tokyo: Kanahara Shuppan 1993
- 5 **Hisamichi S,** Sugawara N. Mass screening for gastric cancer by X-ray examination. *Jpn J Clin Oncol* 1984; **14**: 211-223
- 6 **Maehara Y,** Okuyama T, Oshiro T, Baba H, Anai H, Akazawa K, Sugimachi K. Early carcinoma of the stomach. *Surg Gynecol Obstet* 1993; **177**: 593-597
- 7 **Sano T,** Kobori O, Muto T. Lymph node metastasis from early gastric cancer: endoscopic resection of tumour. *Br J Surg* 1992; **79**: 241-244
- 8 **Maehara Y,** Orita H, Okuyama T, Moriguchi S, Tsujitani S, Korenaga D, Sugimachi K. Predictors of lymph node metastasis in early gastric cancer. *Br J Surg* 1992; **79**: 245-247
- 9 **Yamao T,** Shirao K, Ono H, Kondo H, Saito D, Yamaguchi H. Risk factors for lymph node metastasis from intramucosal gastric carcinoma. *Cancer* 1996; **77**: 602-606
- 10 **Tsujitani S,** Oka S, Saito H, Kondo A, Ikeguchi M, Maeta M, Kaibara N. Less invasive surgery for early gastric cancer based on the low probability of lymph node metastasis. *Surgery* 1999; **125**: 148-154
- 11 **Folli S,** Morgagni P, Roviello F, De Manzoni G, Marrelli D, Saragoni L, Di Leo A, Gaudio M, Nanni O, Carli A, Cordiano C, Dell' Amore D, Vio A. Risk factors for lymph node metastases and their prognostic significance in early gastric cancer (EGC) for the Italian Research Group for Gastric Cancer (IRGGC). *Jpn J Clin Oncol* 2001; **31**: 495-499
- 12 **Sowa M,** Kato Y, Nishimura M, Kubo T, Maekawa H, Umeyama K. Surgical approach to early gastric cancer with lymph node metastasis. *World J Surg* 1989; **13**: 630-636
- 13 **Kitamura K,** Yamaguchi T, Taniguchi H, Hagiwara A, Sawai K,

- Takahashi T. Analysis of lymph node metastasis in early gastric cancer: Rationale of limited surgery. *J Surg Oncol* 1997; **64**: 42-47
- 14 **Kurihara N**, Kubota T, Otani Y, Ohgami M, Kumai K, Sugiura H, Kitajima M. Lymph node metastasis of early gastric cancer with submucosal invasion. *Br J Surg* 1998; **85**: 835-839
- 15 **Mita T**, Shimoda T. Risk factors for lymph node metastasis of submucosal invasive differentiated type gastric carcinoma: clinical significance of histological heterogeneity. *J Gastroenterol* 2001; **36**: 661-668
- 16 **Shimada S**, Yagi Y, Honmyo U, Shiomori K, Yoshida N, Ogawa M. Involvement of three or more lymph nodes predicts poor prognosis in submucosal gastric carcinoma. *Gastric Cancer* 2001; **4**: 54-59
- 17 **Seto Y**, Nagawa H, Muto T. Impact of lymph node metastasis on survival with early gastric cancer. *World J Surg* 1997; **21**: 186-190
- 18 **Nio Y**, Tsubono M, Kawabata K, Masai Y, Hayashi H, Meyer C. Comparison of surgical curves of gastric cancer patients after surgery according to the UICC stage classification and General Rules for Gastric Cancer Study by the Japanese Research Society for gastric cancer. *Ann Surg* 1993; **218**: 47-53
- 19 **Moreaux J**, Bougaran J. Early gastric cancer: a 25-year surgical experience. *Ann Surg* 1993; **217**: 347-355
- 20 **Yasuna O**, Hayashi S. Factors influencing the postoperative course 113 patients with early gastric cancer. *Jpn J Clin Oncol* 1986; **16**: 325-334
- 21 **Ichikura T**, Uefuji K, Tomimatsu S, Okusa Y, Yahara T, Tamakuma S. Surgical strategy for patients with gastric carcinoma with submucosal invasion. *Cancer* 1995; **76**: 935-940
- 22 **Viste A**, Svanes K, Janssen CW, Maartmann-Moh H, Soreide O. Prognostic importance of radical lymphadenectomy in curative resections for gastric cancer. *Eur J Surg* 1994; **160**: 497-502
- 23 **Abe N**, Watanabe T, Suzuki K, Machida M, Toda H, Nakaya Y, Masaki T, Mori T, Sugiyama M, Atomi Y. Risk factors predictive of lymph node metastasis in depressed early gastric cancer. *Am J Surg* 2002; **183**: 168-172
- 24 **Baba H**, Maehara Y, Okuyama T, Orita H, Anai H, Akazawa K, Sugimachi K. Lymph node metastasis and macroscopic features in early gastric cancer. *Hepatogastroenterology* 1994; **41**: 380-383
- 25 **Wu CY**, Chen JT, Chen GH, Yeh HZ. Lymph node metastasis in early gastric cancer: a clinicopathological analysis. *Hepatogastroenterology* 2002; **49**: 1465-1468
- 26 **Sasaki O**, Kido K, Nagahama S. DNA ploidy, Ki-67 and p53 as indicators of lymph node metastasis in early gastric cancer. *Anal Quant Cytol Histol* 1999; **21**: 85-88

Edited by Wang XL Proofread by Zhu LH

# Effect of human milk and colostrum on *Entamoeba histolytica*

Ciler Akisu, Umit Aksoy, Hasan Cetin, Sebnem Ustun, Mete Akisu

**Ciler Akisu, Umit Aksoy**, Department of Parasitology, School of Medicine, Dokuz Eylul University, 35340, Inciralti, Izmir, Turkey  
**Hasan Cetin, Mete Akisu**, Department of Pediatrics, School of Medicine, Ege University, 35100, Bornova, Izmir, Turkey  
**Sebnem Ustun**, Department of Gastroenterology, School of Medicine, Ege University, 35100, Izmir, Turkey  
**Correspondence to:** Ciler Akisu, MD, Department of Parasitology, School of Medicine, Dokuz Eylul University, 35340 Inciralti-Izmir, Turkey. ciler.akisu@deu.edu.tr  
**Telephone:** +90-232-412 45 40 **Fax:** +90-232-259 05 41  
**Received:** 2003-10-15 **Accepted:** 2003-12-16

## Abstract

**AIM:** Many defense factors of the mother's colostrum or milk protect infants from intestinal, respiratory and systemic infections. In the present study, we investigated the effect of colostrum and mature human milk on *E. histolytica* parasites *in vitro*.

**METHODS:** Samples of human milk were collected from 5 healthy lactating mothers. The medium with human milk at concentrations of 2%, 5% and 10% was obtained.

**RESULTS:** The lethal effect of *E. histolytica* on the medium supplemented with different concentrations of both colostrum and mature human milk was significant during the first 30 min. We also detected that the results of colostrum and mature human milk were similar. No statistically significant differences were found between same concentrations of colostrum and mature human milk at the same times.

**CONCLUSION:** Colostrum and mature human milk have significant lethal effect on *E. histolytica* and protect against its infection in breast fed children.

Akisu C, Aksoy U, Cetin H, Ustun S, Akisu M. Effect of human milk and colostrum on *Entamoeba histolytica*. *World J Gastroenterol* 2004; 10(5): 741-742  
<http://www.wjgnet.com/1007-9327/10/741.asp>

## INTRODUCTION

*Entamoeba histolytica* has a worldwide distribution with a high prevalence in areas with poor hygiene, overcrowding, and low socioeconomic conditions. When ingested, cyst passes through the stomach and excyst in the distal part of the small intestine and colon. *E. histolytica* generally lives in the lumen of the large intestine of man. The emerging trophozoites live in the lumen of the bowel close to the mucosa, where they feed by phagocytosis on particulate matter and bacteria and by pinocytosis on liquid nutrients. As such, they may invade the gut wall and produce ulceration and subsequent dysentery. The parasite may be transported by the blood to extraintestinal locations, such as the liver, lungs, and brain<sup>[1-3]</sup>.

The role of breast milk ingestion in passive and active protection of infants is very important. Some reports showed that the incidence of intestinal and systemic *E. histolytica* infections was decreased in human breast milk fed infants.

This protective effect has been attributed to the anti-infective and anti-inflammatory properties of human breast milk<sup>[4,5]</sup>.

The aim of the present study was to investigate the effects of colostrum and mature human milk on *E. histolytica*. Human colostrum or mature milk, if effective in producing lethal effect on *E. histolytica*, could be useful in preventing infections caused by infected water and food in breast-fed children.

## MATERIALS AND METHODS

*E. histolytica* fresh clinical isolate was obtained from a patient with acute amebiasis. The isolate was cultivated in Robinson medium<sup>[6]</sup>.

Fresh human milk was collected from 5 healthy lactating mothers with no clinical evidence of infection or inflammation, who voluntarily donated up to 10 mL of milk and colostrum samples, with their informed consent, by breast pump (Ameda Egnell SMB Pump, Switzerland), in Ege University Hospital, Izmir, Turkey. None of these mothers received hormones or antibiotics during the postpartum period. Colostrum and mature human milk were obtained during the first 3 d of lactation and the 3<sup>rd</sup> wk of lactation, respectively. All samples were collected from mothers between 9 and 12 a.m., just before they began to nurse their babies. The colostrum and mature human milk samples were rapidly centrifuged for 15 min at 1 000 g. The fresh clear middle layer was collected and immediately frozen (-70 °C) until the study<sup>[7]</sup>.

## Culture methods and evaluation of amoeba-cidal activity

The trophozoites were adapted by three successive subcultures at 36±0.5 °C. At the end of the period amoebae reached logarithmic growth phase. Trophozoites were chilled for 10 min in an ice water bath, then vigorously shaken to detach amoebae adherent to the walls of the tube. The culture tubes were centrifuged for 2 min (500 r/min) and supernatant was discarded. Once their content was homogenized by repeated inversion. They were counted with a haemocytometer and then 20×10<sup>4</sup> amoebae/mL were transferred to fresh medium. Five tubes including 5 mL medium were prepared for each group. Then, colostrum and mature human milk were added in medium at different concentrations (2%, 5% and 10%). Tubes were incubated at 36±0.5 °C for 30, 60, 120, 180 and 240 min respectively. At the end of incubation, only motile parasites were counted with a haemocytometer<sup>[8]</sup>. All experiments were done in triplicate and repeated at least twice.

## Statistical analysis

All values were given as mean±SD. To determine the statistical significance of the difference between study groups, we used nonparametric tests, Kruskal-Wallis analysis of variance and Mann-Whitney test. *P* value less than 0.05 was considered significant.

## RESULTS

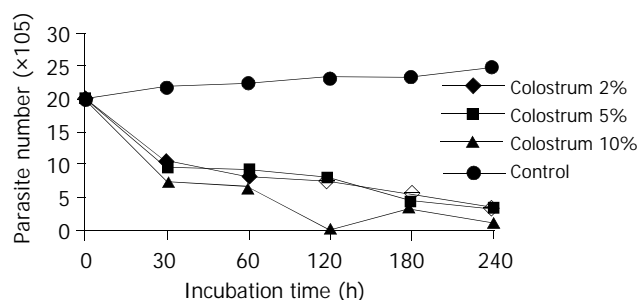
In the study, we analyzed the lethal effect of colostrum and mature human milk at three different concentrations on *E. histolytica*. The number of living parasite was recorded at the different incubation periods. The results of this experiment showed that the best lethal effect (50% killing) was obtained from all samples

during the first 30 min exposure. We have also observed that there was no living parasite at the end of 12 h incubation.

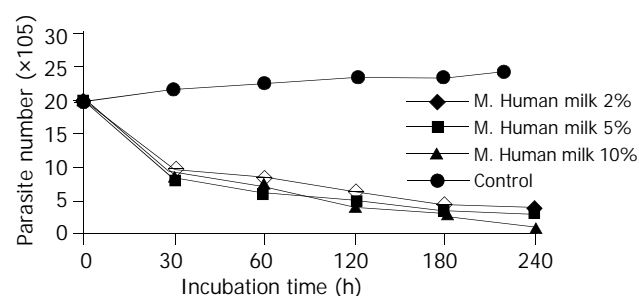
A significant lethal effect of different concentrations of colostrum on *E. histolytica* was observed during 30 min and 180 min exposure ( $P < 0.001$ ) (Figure 1). However, no statistically significant differences were shown between them ( $P > 0.05$ ).

When the lethal effect of mature human milk at different concentrations on *E. histolytica* was compared with each other, the difference was statistically significant during 30 min and 180 min exposure ( $P < 0.001$ ) (Figure 2). There was no statistically significant difference between 30 and 180 min exposure ( $P > 0.005$ ).

The results of colostrum and mature human milk were similar. Colostrum did not prove to be very superior to mature human milk. No statistically significant differences were found between same concentrations of colostrum and mature human milk at 30, 60, 120, 180, and 240 min exposure.



**Figure 1** Lethal effect of colostrum at various concentrations on *E. histolytica*.



**Figure 2** Lethal effect of mature human milk at various concentrations on *E. histolytica*.

## DISCUSSION

Colostrum and mature human milk may play a protective role in breast-fed children exposed to some parasites (such as *G. intestinalis*). Both contain considerable amounts of immunoglobulins, mainly the secretory immunoglobulin A (sIgA) type that may play a role in such protection. It has been shown that such immunoglobulins have antibody specificities, which reflect antigenic stimuli in the intestinal tract<sup>[9,10]</sup>.

The demonstration of antibodies in breast milk may have epidemiological significance in population studies. Antibodies of the IgA class were found in serum and colostrum of parturient women in an endemic area of amebiasis<sup>[11]</sup>. Grundy *et al*<sup>[12]</sup> demonstrated sIgA antibodies to *E. histolytica* in milk (31%) and serum (14%) of mothers living in Kenya. They suggested that serum antibodies indicated past or present invasive amoebiasis, milk antibodies were more likely to present intestinal infection in endemic areas. Anti-Eh sIgA antibody titers were significantly increased in colostrum samples of mothers of newborn children with diarrhea<sup>[13]</sup>. The infants,

mostly uninfected, were found to have *E. histolytica* cysts in small numbers (2/1 200 samples), despite the high prevalence of *E. histolytica* in their mothers<sup>[14]</sup>.

Human milk cells (macrophages, lymphocytes, neutrophils) and antibodies could protect intestinal mucosa, remain active in the neonatal intestine and possibly migrate to other tissues<sup>[5]</sup>. Colostral macrophages might be cytotoxic to trophozoites of *E. histolytica*. This has been shown by direct microscopy<sup>[7]</sup>. It was proposed that colostral macrophages might interrupt colonization and subsequent invasion in infants who were breast-fed. Although there are many epidemiological studies in endemic areas, studies about colostrum and mature human milk *in vitro* are limited. Gillin *et al* reported that 90% of *E. histolytica* were killed in approximately 3 h with 1% milk. But we could not find any publications containing colostrum and mature human milk together *in vitro*. We established that *E. histolytica* was killed by exposure to colostrum and mature human milk *in vitro*. IgA and lactoferrin amounts in colostrum were richer than mature human milk. However, we obtained similar results from both of them. Fifty percent of *E. histolytica* were killed by all dilutions of both secretions during a 30 min exposure. Thus, the lethal effect did not depend on the amounts of various protectable milk components. Similarly, some studies showed that the lethal effect of sIgA on some parasites did not depend on the amount of sIgA.

In this study, we showed that colostrum and mature human milk had direct lethal effect on *E. histolytica* *in vitro*. Therefore, when trophozoites that emerge from cysts in the small intestine are exposed to colostrum and mature human milk, they may be killed. Thus, breast fed children have a lower rate of amebiasis than nonbreast fed children.

## REFERENCES

- 1 Ravdin JJ. Amebiasis. *Clin Infect Dis* 1995; **20**: 1453-1464
- 2 Proctor EM. Laboratory diagnosis of amebiasis. *Clin Lab Med* 1991; **11**: 829-859
- 3 Stanley SL Jr. Amoebiasis. *Lancet* 2003; **22**: 1025-1034
- 4 Chierici R. Antimicrobial actions of lactoferrin. *Adv Nutr Res* 2001; **10**: 247-269
- 5 Xanthou M, Bines J, Walker WA. Human milk and intestinal host defense in newborns: an update. *Adv Pediatr* 1995; **42**: 171-208
- 6 Clark CG, Diamond LS. Methods for cultivation of luminal parasitic protists of clinical importance. *Clin Microbiol Rev* 2002; **15**: 329-341
- 7 Acosta-Altamirano G, Rocha-Ramirez LM, Reyes-Montes R, Cote V, Santos JJ. Anti-amoebic properties of human colostrum. *Adv Exp Med Biol* 1987; **216B**: 1347-1352
- 8 Chavez-Duenas L, Gomez-Dominguez R, Lopez-Revilla R. Effects of Panmede and various horse serum concentrations on the axenic cultivation of *Entamoeba histolytica* strains in TPS-1 medium. *Parasitol Res* 1989; **76**: 50-54
- 9 Hanson LA, Korotkova M. The role of breastfeeding in prevention of neonatal infection. *Semin Neonatol* 2002; **7**: 275-281
- 10 Ogra PL, Losonsky GA, Fishaut M. Colostrum-derived immunity and maternal-neonatal interaction. *Ann N Y Acad Sci* 1983; **409**: 82-95
- 11 Berber AC, Escobar A, Zamora M, Acosta G. Identification of *Entamoeba histolytica* antigens recognized by IgA class human antibodies in sera and colostrum of puerperal women using immunoblotting techniques. *Arch Invest Med* 1990; **21**(Suppl 1):97-101
- 12 Grundy MS, Cartwright-Taylor L, Lundin L, Thors C, Hult G. Antibodies against *Entamoeba histolytica* in human milk and serum in Kenya. *Clin Microbiol* 1983; **17**: 753-758
- 13 Lopez-Revilla R, Navarro-Garcia F, Valadez-Sanchez M, Lopez Vidal Y, Calva Mercado J. Dot-enzyme-linked immunosorbent assay (Dot-ELISA) of anti-*Entamoeba histolytica* antibodies in human serum and colostrum. *Arch Invest Med* 1991; **22**: 249-253
- 14 Islam A, Stoll BJ, Ljungstrom I, Biswas J, Nazrul H, Hult G. The prevalence of *Entamoeba histolytica* in lactating women and in their infants in Bangladesh. *Trans R Soc Trop Med Hyg* 1988; **82**: 99-103



# Detection of K-ras gene mutation in fecal samples from elderly large intestinal cancer patients and its diagnostic significance

Jun Wan, Zi-Qi Zhang, Wei-Di You, Hua-Kui Sun, Jian-Ping Zhang, Ya-Hong Wang, Yong-He Fu

**Jun Wan, Zi-Qi Zhang, Wei-Di You, Hua-Kui Sun, Jian-Ping Zhang, Ya-Hong Wang, Yong-He Fu**, Department of Geriatric Gastroenterology, General Hospital of the Chinese PLA, Beijing 100853, China

**Correspondence to:** Jun Wan, Department of Geriatric Gastroenterology, South Building of General Hospital of the Chinese PLA, Beijing, China. wanjun@301hospital.com.cn

**Telephone:** +86-10-66937622

**Received:** 2003-11-04 **Accepted:** 2003-12-29

## Abstract

**AIM:** To study the diagnostic significance of K-ras gene mutations in fecal samples from elderly patients with large intestinal cancer.

**METHODS:** DNA was extracted in the fecal and tissue samples from 23 large intestinal cancer patients, 20 colonic adenomatoid polypus patients and 20 healthy subjects. The K-ras gene mutations at the first and second bases of codon 12 were detected by the allele specific mismatch method.

**RESULTS:** The K-ras gene mutation was 56.52%(13/23) in the large intestinal cancer patients, which was notably higher than that in the normal subjects whose K-ras gene mutation was 5%(1/20) ( $\chi^2=12.93$ ,  $P<0.001$ ). There was no significant difference in comparison with that of colonic adenomatoid polypus patients whose K-ras gene mutation was 30%(6/12) ( $\chi^2=3.05$ ,  $P>0.05$ ). The K-ras gene mutation at the second base of codon 12 was 92.13%(12/13) in the large intestinal cancer patients. There was no significant difference between the detection rate of K-ras gene mutation in the fecal and tissue samples ( $\chi^2=9.35$ ,  $P<0.01$ ).

**CONCLUSION:** Our results indicate that detection of the K-ras gene mutations in fecal samples provides a non-invasive diagnostic method for the elderly large intestinal cancer patients. Its significance in the early diagnosis of large intestinal cancer awaits further studies.

Wan J, Zhang ZQ, You WD, Sun HK, Zhang JP, Wang YH, Fu YH. Detection of K-ras gene mutation in fecal samples from elderly large intestinal cancer patients and its diagnostic significance. *World J Gastroenterol* 2004; 10(5): 743-746  
<http://www.wjgnet.com/1007-9327/10/743.asp>

## INTRODUCTION

Large intestinal cancer is one of the common malignant tumors in China. Its incidence has been increasing in the elderly, and its death rate is approximately 60% in large intestinal cancer patients over 60 years old. In China, large intestinal cancer is often resulted from the malignancy of colonic adenomas, because the incidence of large intestinal polypus is high in the elderly. It was reported that the detectable rate of large intestinal polypus and adenomatoid polypus was as high as 62.1% and 67.9%, respectively<sup>[1]</sup>. Therefore, early detection of cancerous

adenomas is of great significance in decreasing the incidence and death rate of large intestinal cancer. At present, colonoscopy is the most ideal diagnostic method for large intestinal cancer<sup>[2,3]</sup>, the reported detectable rate of early large intestinal cancer was 36.5% in the elderly<sup>[1]</sup>. Since colonoscopy is an invasive method and the examined subjects would have some suffering, it has therefore become a topic of general interest to find a non-invasive diagnostic method for large intestinal cancer patients<sup>[4-10]</sup>. The K-ras gene mutations in fecal samples from the elderly were detected by the allele specific mismatch method, and its diagnostic significance in the large intestinal cancer patients was discussed.

## MATERIALS AND METHODS

### Reagents

Taq DNA polymerase, dNTPS, DNA fragments, agarose, DNA extraction kits were the products of Promega (Madison, USA). Proteinase K was the product of Merck.

### Specimens

The patients enrolled in this study were 23 cases of large intestinal cancer (19 males, 4 females, averaging 68.8 years), 20 cases of colonic adenomatoid polypus and 20 healthy subjects. Their diagnoses were confirmed by endoscopy and biopsy. Of the 23 cases of large intestinal cancer, 5 had well differentiated adenocarcinomas, 10 had moderately differentiated adenocarcinomas, 6 had poorly differentiated adenocarcinomas, and 2 had mucinous adenocarcinomas. The fecal samples were collected from the above patients before undergoing surgery and stored at -30 °C.

### DNA extraction

DNA was extracted from the fecal samples using the DNA extraction kits. The fecal samples were processed according to the following procedures: 100-200 g of the fecal samples was diluted in 500  $\mu$ L of phosphate buffered saline (PBS), pH7.5, and homogenized for 2 min at 1 000 r/min. Then, 500  $\mu$ L supernatant after the addition of 50  $\mu$ L hydrolytic buffer was homogenized for 5 min, and centrifuged at 6 000 r/min for 2 min. The precipitate was placed into 500  $\mu$ L cleaning solution and centrifuged at 6 000 r/min for 2 min. After washed twice and addition of 50  $\mu$ L lysate and covered by paraffin oil, the precipitate was boiled for 5 min, centrifuged at 13 000 g for 10 min and stored at -30 °C before it was used. The DNA in the 16 cancer tissue samples was extracted by proteinase K (10 g/L, thermostatic water bath at 37 °C for 24 h), and purified by phenol-chloro-isopentanol extraction, and dissolved in TE buffer after ethanol precipitation for use.

### PCR reaction

The oligonucleotide primer was synthesized by the Oligo 1000DNA synthesizer (Beckman). The DNA amplification PCR reaction was carried out in a total volume of 50  $\mu$ L buffer containing 5  $\mu$ L of diluted DNA templates, 10 mmol/L Tris-HCl (pH8.8), 1.5 mmol/L MgCl<sub>2</sub>, 1g/L Triton X-100, 200 mmol/L dNTPs, 50 pmol/L of each primer and 1U of Taq DNA

polymerase. The PCR conditions were as follows: denaturing at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 3 min. The amplification was performed for 30 cycles. L14841 and H15149 are the primers specific for human cytochrome B gene, COI and ND2 are the primers specific for human mitochondrial cytochrome oxidase COI subunit. These primers were also used in the detection of K-ras gene mutations.

The point mutation at the first and second bases of codon 12 of the K-ras gene was detected by the allele specific mismatch method<sup>[11]</sup>. The amplification PCR reaction was performed for 45 cycles in a total volume of 50 µL of buffer, but the conditions were slightly modified as the follows: denaturing at 95 °C for 1 min, annealing at 58 °C for 2 min, extension at 72 °C for 1 min. The A set and B set primers were used to detect the presence of K-ras gene mutations at the first and second bases of codon 12. Besides one general primer found in the 2 groups, both Ag and Bc primers were specific for the wild-type K-ras gene. The other primers were used to amplify various mutations of the K-ras gene. The control bands of DNA fragments from the corresponding tumors were validated using an ultraviolet detector after the PCR products were analyzed on a 30g/L agarose gel and visualized by ethidium bromide staining.

The nucleotide sequences of human cytochrome B gene primers and human mitochondrial cytochrome oxidase COI subunits used in the amplification reactions were as follows: L14841: 5' AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA3', H15 149: 5' AAAGTGCAGCCCCCTCAGAATGATATTTGTCCTCA3', COI: 5' ACGATGTCTAGTGATGAGTTGCTA3', ND2: 5' ACGCCTAATCTACTCCACCTCAATC 3'.

A 300-bp PCR amplification product was detected using L14841 and H15149, and a 1 400 bp PCR amplification product was detected using COI and ND2. The K-ras gene mutation detected at the first base of codon 12 using the A set primers included K-ras A: 5' CAGAGAAACCTTTATCTG 3', K-ras Aa: 5' TGGTAGTTGGAGCTA 3'. K-ras Ac, K-ras Ag and K-ras At differed from K-ras Aa in the last nucleotide, which was replaced by C, G and T respectively<sup>[11]</sup>, and produced a 146 bp DNA amplification fragment.

The K-ras gene mutations detected using the B set primers at the second base of codon 12 included K-ras B: 5' GTACTGGTGGAGTATTT3' and K-ras Ba: 5' ACTCTTGCCTACGCCAA3'. K-ras Bc, K-ras Bg and K-ras Bt differed from K-ras Ba in the last 3' nucleotide, which was replaced by C, G and T respectively<sup>[11]</sup>, and generated a 161 bp DNA amplification fragment.

## RESULTS

### Sequence of specific human DNA in fecal extraction

The DNA of human cytochrome B gene (a 300 bp amplification fragment) and human mitochondrial cytochrome oxidase COI (a 1 400 bp amplification fragment) in the extraction of 10 fecal samples was amplified using the pair of L14841 and H15149 primers and the pair of COI and ND2 primers. A 300 bp and a 1 400 bp electrophoretic bands were observed in 9 fecal samples after the PCR products were analyzed on a 15g/L agarose gel and visualized by ethidium bromide staining.

### Consistency of K-ras gene mutations in fecal and tissue samples

The K-ras gene mutations in the fecal and tissue samples of 16 large intestinal cancer patients were detected. Of the 16 large intestinal cancer patients, 9 had identical K-ras gene mutation detected both in fecal and tissue samples, none had K-ras gene mutation detected both in fecal and tissue samples, and only 2 had K-ras gene mutation in tissue samples. The consistency test showed that the K-ras gene mutations in fecal and tissue samples were well correlated ( $\chi^2=9.35$ ,  $P<0.01$ ).

### K-ras gene mutation in fecal samples

The K-ras gene mutation was detected in the fecal samples from 23 large intestinal cancer patients. The K-ras gene mutation rate was 56.25% (13/23), which was significantly higher than that (5%, 1/20) in the healthy subjects ( $\chi^2=12.93$ ,  $P<0.001$ ). There was no significant difference in comparison with that (30%, 6/20) of colonic adenomatoid polypi ( $\chi^2=3.05$ ,  $P>0.05$ ). The K-ras gene mutation rate was 40% (2/5), 60% (6/10), 66.67% (4/6), and 50% (1/2) in the well, moderately and poorly differentiated adenomas and mucinous adenomas, respectively. The K-ras gene mutation was detected in 2 patients with cancerous colonic adenomatoid polypus, its mutation rate was 30% (6/20) in the fecal samples from colonic adenomatoid polypus patients, which was significantly higher than that in the healthy subjects ( $\chi^2=4.33$ ,  $P<0.05$ ). The mutation rate of K-ras gene was 23.08% (3/13), 40% (2/5), and 50% (1/2) in the polypi with a diameter less than 1 cm, a diameter of 1-2 cm, a diameter larger than 2 cm, respectively. Among the 13 large intestinal cancer patients, the K-ras gene mutation was detected at the second base of codon 12 in 12 patients (92.31%), GGT was mutated into GAT and GTT in 9 and 3 patients, respectively. The K-ras gene mutation site was observed at the first base of codon 12 in 1 patient, whose GGT was mutated into GTT.

## DISCUSSION

A healthy adult excretes approximately  $10^{10}$  epithelial cells every day. A large number of tumor cells will renew and exfoliate into the intestinal cavity of colonic cancer patients every day. A certain amount of DNA can maintain its stability due to the resistance of intestinal tumor cells to various degradation enzymes or due to the impairment of apoptotic mechanism of tumor cells<sup>[12]</sup>. Based on the above findings, Sidransky *et al*<sup>[13]</sup> detected the K-ras gene mutation in the fecal samples from early large intestinal cancer patients in 1992, and found the K-ras gene mutation in the fecal and tissue samples from tumor patients. Since then, several scholars have carried out some similar studies<sup>[11,14-16]</sup>. However, their research findings have not been popularized and applied due to low PCR amplification rate of DNA in fecal samples. We used the conventional phenol-chloroform extraction method to extract DNA in 10 fecal samples. The PCR amplification product was observed only in 3 faecal samples using the primers specific for mitochondrial DNA of human eukaryotic cells. Then, the PCR amplification reaction was performed in the DNA extraction kits, the amplification rate was as high as 90% (9/10) when the DNA extraction kits used in the detection of DNA in fecal samples were washed twice in hydrolysate to remove the hybrid proteins. In the experiment, we found that the number of templates had a certain effect on the PCR amplification reaction, and could dilute the extract stock to some extent. The results indicated that the amplification rate increased with increase of dilution strength, suggesting that the amount of DNA was quite suitable to its amplification. The effect of some PCR inhibitors present in the PCR amplification reaction was significantly reduced due to the increase of dilution strength. Berndt *et al*<sup>[17]</sup> held that these PCR inhibitors were the bile salts and bilirubin present in the fecal samples. Villa *et al*<sup>[14]</sup> recovered the purified DNA using purification columns after the absorbent was added to the extract from the fecal samples, and found that the amplification rate was significantly increased, but the cost was rather high. In our experiment, the amplification rate was increased when the DNA extract was approximately diluted, which simplified the procedures of DNA preparation and reduced the cost. However, as the amount of templates in the DNA extract stock from the fecal samples was uncertain, its dilution factor exerted an effect on the DNA

amplification stability. Therefore, the fecal samples with a poor amplification result should be amplified again after the dilution factor of the extract stock was adjusted. This would no doubt increase the cost and time in detecting some fecal samples. In the present study, we detected the K-ras gene mutation in tissue and fecal samples from 16 large intestinal cancer patients, and achieved a rather good consistency ( $P < 0.01$ ), indicating that detection of the K-ras gene mutation could reflect the presence of its mutation in the tissues. Since it is much easier to obtain tissue samples than fecal samples in clinic, this method will make it clinically possible to screen colorectal cancer.

Researches showed that the K-ras gene mutation in oncogenes was most frequently found in large intestinal cancer, accounting for 40-50%<sup>[18-20]</sup>. Smith-Ravin *et al.*<sup>[11]</sup> detected the K-ras gene mutation in the fecal samples from large intestinal cancer patients using the allele specific mismatch method, and found that the mutation rate was 50%. Xiao *et al.*<sup>[21]</sup> detected the K-ras gene mutation in the fecal samples from large intestinal cancer patients using PCR-RFLP, and found the mutation rate was 36.4%. In our study, the detection rate of K-ras gene mutation was 56.25% and 5% in the fecal samples from large intestinal cancer patients and healthy subjects, respectively. There was a very significant difference between the two groups ( $P < 0.001$ ), but there was no significant difference in comparison with that of colonic adenomatoid polypus patients ( $P > 0.05$ ), suggesting that adenomatoid polypus, a precancerous lesion of large intestinal cancer, is closely related with the development of colonic cancer. According to the literature reports, 90% of large intestinal cancers were resulted from large intestinal adenomatoid polypi. Therefore, early detection and resection of large intestinal adenomatoid polypi could greatly reduce the incidence of large intestinal cancer<sup>[2,22]</sup>. However, the diagnostic significance of large intestinal cancer at its early stages should be further studied in a larger number of large intestinal cancer patients. Many researchers<sup>[23-29]</sup> held that the mutation rate of K-ras gene in colonic adenomas would increase with the growth of adenomas. In our study, among the two cases of adenomas with a diameter larger than 2 cm, the K-ras gene mutation was detected in one case, and was also detected in the 2 cases after their colonic adenomatoid polypi were found to be cancerous. This was consistent with the K-ras gene mutation found at the early stages of large intestinal cancer reported both in Chinese and foreign literature. Further researches showed that the K-ras gene mutation was usually resulted from the mutation of GGT to GAT of its codon 12, and 84% K-ras gene mutations were observed at the second base of codon 12. In our study, the mutation site of K-ras gene was observed at the second base of codon 12, accounting for 92.31% (12/13), 69.23% of which was resulted from the mutation of GGT to GAT, and 23.08% was resulted from the mutation of GGT to GTT. This was consistent with that reported in the literature<sup>[11,30,31]</sup>.

The non-invasive method for the detection of K-ras gene mutation in fecal samples reported in this paper is simple to operate and the samples are easy to collect. Its preliminary application in clinic has shown that it is of significance in the diagnosis of elderly large intestinal cancer patients, and can be used in combination with colonoscopy to screen the high risk population for colorectal cancer. It should be further improved because its PCR reaction time is long, which leads to the prolonged detection and is disadvantageous to the detection of large samples.

## REFERENCES

- 1 **Wan J**, Zhang ZQ, Zhu C, Wang MW, Zhao DH, Fu YH, Zhang JP, Wang YH, Wu BY. Colonoscopic screening and follow-up for colorectal cancer in the elderly. *World J Gastroenterol* 2002; **8**: 267-269
- 2 **Pignone M**, Rich M, Teutsch SM, Berg AO, Lohr KN. Screening for colorectal cancer in adults at average risk: a summary of the evidence for the U.S. Preventive Services Task Force. *Ann Intern Med* 2002; **137**: 132-141
- 3 **Xiang DN**, Xiang P, Zheng SB, Cao XY, Wang GS. Diagnostic value of colonofibroscopy in 1507 elderly patients of lower gastrointestinal bleeding. *Laonian Yixue Yu Baojian* 2002; **8**: 35-36
- 4 **Atkin W**. Options for screening for colorectal cancer. *Scand J Gastroenterol Suppl* 2003; **237**: 13-16
- 5 **Meng W**, Li SR, Li L. Effect of the combined sequent program for screening colorectal carcinoma. *Linchuang Xiaohuabin Zazhi* 2000; **11**: 157-158
- 6 **Lou CY**, Li SY, Zhu XG. The detection of the rearrangements of bcl-2 gene in the cancer tissues and stool of the patients with colorectal carcinoma by semi-nest PCR. *Zhonghua Shiyan Waikexue* 1999; **16**: 197-198
- 7 **Fan RY**, Li SR, Wu ZT. The detection of K-ras mutations in stools and tissue samples from patients with colorectal cancer. *Zhonghua Xiaohua Zazhi* 2001; **21**: 445-446
- 8 **Fan RY**, Li SR, Wu X, Wu ZT, Chen ZM, Deng YJ, Cao JB, Zhang HG. The expression of P53 in shedding cells from patients with colorectal cancer. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 814-815
- 9 **Mandel JS**, Church TR, Bond JH, Ederer F, Geisser MS, Mongin SJ, Snover DC, Schuman LM. The effect of fecal occult-blood screening on the incidence of colorectal cancer. *N Engl J Med* 2000; **343**: 1603-1607
- 10 **Ito S**, Hibi K, Nakayama H, Kodera Y, Ito K, Akiyama S, Nakao A. Detection of tumor DNA in serum of colorectal cancer patients. *Jpn J Cancer Res* 2002; **93**: 1266-1269
- 11 **Smith-Ravin J**, England J, Talbot IC, Bodmer W. Detection of c-Ki-ras mutations in faecal samples from sporadic colorectal cancer patients. *Gut* 1995; **36**: 81-86
- 12 **Ratto C**, Flamini G, Sofo L, Nucera P, Ippoliti M, Curigliano G, Ferretti G, Sgambato A, Merico M, Doglietto GB, Cittadini A, Crucitti F. Detection of oncogene mutation from neoplastic colonic cells exfoliated in feces. *Dis Colon Rectum* 1996; **39**: 1238-1244
- 13 **Sidransky D**, Tokino T, Hamilton SR, Kinzler KW, Levin B, Frost P, Vogelstein B. Identification of ras oncogene mutations in the stool of patients with curable colorectal tumors. *Science* 1992; **256**: 102-105
- 14 **Villa E**, Dugani A, Rebecchi AM, Vignoli A, Grottola A, Buttafoco P, Losi L, Perini M, Trande P, Merighi A, Lerose R, Manenti F. Identification of subjects at risk for colorectal carcinoma through a test based on K-ras determination in the stool. *Gastroenterology* 1996; **110**: 1346-1353
- 15 **Tagore KS**, Lawson MJ, Yucaitis JA, Gage R, Orr T, Shuber AP, Ross ME. Sensitivity and specificity of a stool DNA multitarget assay panel for the detection of advanced colorectal neoplasia. *Clin Colorectal Cancer* 2003; **3**: 47-53
- 16 **Ito Y**, Kobayashi S, Taniguchi T, Kainuma O, Hara T, Ochiai T. Frequent detection of K-ras mutation in stool samples of colorectal carcinoma patients after improved DNA extraction: comparison with tissue samples. *Int J Oncol* 2002; **20**: 1263-1268
- 17 **Berndt C**, Haubold K, Wenger F, Brux B, Muller J, Bendzko P, Hillebrand T, Kottgen E, Zanow J. K-ras mutations in stools and tissue samples from patients with malignant and nonmalignant pancreatic diseases. *Clin Chem* 1998; **44**: 2103-2107
- 18 **Okulczyk B**, Piotrowski Z, Kowalcuk O, Niklinski J, Chyczewski L. Evaluation of K-RAS gene in colorectal cancer. *Folia Histochem Cytobiol* 2003; **41**: 97-100
- 19 **van Engeland M**, Roemen GM, Brink M, Pachen MM, Weijenberg MP, de-Bruine AP, Arends JW, van den Brandt PA, de Goeij AF, Herman JG. K-ras mutations and RASSF1A promoter methylation in colorectal cancer. *Oncogene* 2002; **21**: 3792-3795
- 20 **Kislitsin D**, Lerner A, Rennert G, Lev Z. K-ras mutations in sporadic colorectal tumors in Israel: unusual high frequency of codon 13 mutations and evidence for nonhomogeneous representation of mutation subtypes. *Dig Dis Sci* 2002; **47**: 1073-1079
- 21 **Xiao SX**, Xu AM, Wang DB, Wu L, Liu HP, Zeng B. The detection of codon 12 mutations of K-ras gene in feces by nested PCR-RFLP. *Zhongguo Shengwu Zhipin Zazhi* 1998; **11**: 103-105

- 22 **Hermesen M**, Postma C, Baak J, Weiss M, Rapallo A, Sciutto A, Roemen G, Arends JW, Williams R, Giaretti W, De Goeij A, Meijer G. Colorectal adenoma to carcinoma progression follows multiple pathways of chromosomal instability. *Gastroenterology* 2002; **123**: 1109-1119
- 23 **Scott N**, Bell SM, Sagar P, Blair GE, Dixon MF, Quirke P. p53 expression and K-ras mutation in colorectal adenomas. *Gut* 1993; **34**: 62162-62164
- 24 **Blum HE**. Colorectal cancer: future population screening for early colorectal cancer. *Eur J Cancer* 1995; **31A**: 1369-1672
- 25 **Hosaka S**, Aoki Y, Akamatsu T, Nakamura N, Hosaka N, Kiyosawa K. Detection of genetic alterations in the p53 suppressor gene and the K-ras oncogene among different grades of dysplasia in patients with colorectal adenomas. *Cancer* 2002; **94**: 219-227
- 26 **Rashid A**, Houlihan PS, Booker S, Petersen GM, Giardiello FM, Hamilton SR. Phenotypic and molecular characteristics of hyperplastic polyposis. *Gastroenterology* 2000; **119**: 323-332
- 27 **Lamlum H**, Papadopoulou A, Ilyas M, Rowan A, Gillet C, Hanby A, Talbot I, Bodmer W, Tomlinson I. APC mutations are sufficient for the growth of early colorectal adenomas. *Proc Natl Acad Sci U S A* 2000; **97**: 2225-2228
- 28 **Rashid A**, Zahurak M, Goodman SN, Hamilton SR. Genetic epidemiology of mutated K-ras proto-oncogene, altered suppressor genes, and microsatellite instability in colorectal adenomas. *Gut* 1999; **44**: 826-833
- 29 **Glarakis IS**, Savva S, Spandidos DA. Activation of the ras genes in malignant and premalignant colorectal tumors. *Oncol Rep* 1998; **5**: 1451-1454
- 30 **Nishikawa T**, Maemura K, Hirata I, Matsuse R, Morikawa H, Toshina K, Murano M, Hashimoto K, Nakagawa Y, Saitoh O, Uchida K, Katsu K. A simple method of detecting K-ras point mutations in stool samples for colorectal cancer screening using one-step polymerase chain reaction/restriction fragment length polymorphism analysis. *Clin Chim Acta* 2002; **318**: 107-112
- 31 **Prix L**, Uciechowski P, Bockmann B, Giesing M, Schuetz AJ. Diagnostic biochip array for fast and sensitive detection of K-ras mutations in stool. *Clin Chem* 2002; **48**: 428-435

Edited by Wang XL Proofread by Zhu LH

# Clinical evaluation of four one-week triple therapy regimens in eradicating *Helicobacter pylori* infection

Chuan-Yong Guo, Yun-Bin Wu, Heng-Lu Liu, Jian-Ye Wu, Min-Zhang Zhong

**Chuan-Yong Guo, Yun-Bin Wu, Heng-Lu Liu, Jian-Ye Wu, Min-Zhang Zhong**, Department of Gastroenterology, Tielu hospital of Tongji University, Shanghai 200072, China

**Correspondence to:** Dr. Chuan-Yong Guo, Department of Gastroenterology, Tielu Hospital of Tongji University, Shanghai 200072, China. guochuanyong@hotmail.com

**Telephone:** +86-21-56779971 **Fax:** +86-21-66303983

**Received:** 2003-06-04 **Accepted:** 2003-07-24

## Abstract

**AIM:** To evaluate clinical efficacy of four one-week triple therapies in eradicating *Helicobacter pylori* infection.

**METHODS:** In this clinical trial, 132 patients with duodenal ulcer and chronic gastritis were randomly divided into four groups, and received treatment with OAC (omeprazole 20 mg + amoxicillin 1 000 mg + clarithromycin 250 mg), OFC (omeprazole 20 mg + furazolidone 100 mg + clarithromycin 250 mg), OFA (omeprazole 20 mg + furazolidone 100 mg + amoxicillin 1 000 mg) and OMC (omeprazole 20 mg + metronidazole 200 mg + clarithromycin 250 mg), respectively. Each drug was taken twice daily for one week. The <sup>13</sup>C urea breath test was carried out 4-8 weeks after treatment to determine the success of *H pylori* eradication.

**RESULTS:** A total of 127 patients completed the treatment. The eradication rate for *H pylori* infection was 90.3%, 90.9%, 70.9% and 65.6%, respectively in OAC, OFC OMC and OFA groups.

**CONCLUSION:** A high eradication rate can be achieved with one-week OAC or OFC triple therapy. Thus, one-week triple therapies with OAC and OFC are recommended for Chinese patients with duodenal ulcers and chronic gastritis.

Guo CY, Wu YB, Liu HL, Wu JY, Zhong MZ. Clinical evaluation of four one-week triple therapy regimens in eradicating *Helicobacter pylori* infection. *World J Gastroenterol* 2004; 10(5): 747-749  
<http://www.wjgnet.com/1007-9327/10/747.asp>

## INTRODUCTION

Eradication of *Helicobacter pylori* infection has become a wide clinical practice for *H pylori* related diseases such as peptic ulcers, and considerable clinical efficacy has been achieved over the past two decades<sup>[1-6]</sup>. However, many short-term (one week) triple therapy regimens include metronidazole and suffer from the problem of metronidazole resistance, which could significantly decreases clinical efficacy<sup>[7-11]</sup>. Therefore, it is a very important issue to search for anti-*H pylori* regimens that are highly effective in eradicating *H pylori* infection but without drug resistance<sup>[12]</sup>. The aim of the present study was to evaluate the clinical efficacy of four short-term triple therapy regimens with clarithromycin.

## MATERIALS AND METHODS

### Selection of patients

**Criteria of selection** (1) Those aged 18-70 years. (2) Those with duodenal ulcer (DU) or chronic gastritis (CG) confirmed by gastroscopy. (3) Those who were positive for *H pylori* by a rapid urease test (RUT) and positive by serology, silver or Giemsa staining and histological examination.

**Criteria of exclusion** (1) Patients who had gastric ulcer or severe gastroesophageal reflux disease, and those who had gastric operation history, hemolytic anemia or family history of hemolytic anemia. (2) Patients who were in lactation or pregnancy. (3) Patients who had combined severe diseases of other system that might affect the medical evaluation of this study. (4) Patients who took the drugs included in this study over the past month. (5) Patients who was allergic to the drugs included in this study.

### Methods

**Drugs** Omeprazole (20 mg/cap, Changzhou fourth Pharmaceutical Factory), clarithromycin (250 mg/tab, Hangzhou Chinese-American Eastchina Pharmaceutical Co. Ltd), furazolidone (100 mg/tab, Guangdong Jiangmen Pharmaceutical Factory), metronidazole (200 mg/tab, Shanghai Ensai Pharmaceutical Co. Ltd) and amoxicillin (250 mg/cap, Kunming Baker Norton Pharmaceutical Co. Ltd) were used.

**Regimens** Patients were randomly divided into four groups, and receive treatment with OAC (omeprazole 20 mg + amoxicillin 1 000 mg + clarithromycin 250 mg), OFC (omeprazole 20 mg + furazolidone 100 mg + clarithromycin 250 mg), OMC (omeprazole 20 mg + metronidazole 200 mg + clarithromycin 250 mg) and OFA (omeprazole 20 mg + furazolidone 100 mg + amoxicillin 1 000 mg), respectively. Each group took the drugs twice a day for 7 d.

**Procedures** 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 gastroscopy examination, four biopsy specimens were taken from stomach: one for a rapid urease test (RUT), one for silver or modified Giemsa staining, and two for histological examination. Serum anti-*H pylori* IgG antibodies were also detected. The patients who were intensive positive by the RUT (positive in five minutes) were initially considered to be qualified for the study. Only those patients who were also positive by serology, *H pylori* staining and histological examination were included in the clinical trial. Patients were followed up on the eighth day to check clinical symptoms, side effects and compliance. A <sup>13</sup>C urea breath test was carried out 4-8 wk after completion of the therapy.

**Definition of *H pylori* eradication** *H pylori* eradication was defined when the <sup>13</sup>C urea breath test was negative 4-8 weeks after completion of anti-*H pylori* therapy.

### Statistical analysis

*H pylori* eradication rate was the main analytic target. Total eradication rate and its 95% confidence interval of each regimen was calculated and analyzed by intention-to-treat

analysis (ITT) and per protocol (PP), respectively. The significance in the difference of eradication rate between various regimens was tested by Fisher exact probability and Chi-square test. The possible factors affecting eradication rate was analyzed in a logistic regression model. The difference in the incidence of side effects of each regimen was tested by Fisher exact probability test.

## RESULTS

### Demographic and clinical data

Of the 132 patients enrolled in the study, 127 (96.2%) completed the treatment and five (3.8%) dropped off. The demographic data and the proportion of DU and CG were not significantly different among the groups (Table 1).

**Table 1** Comparison between patient age gender and endoscopic diagnostic results of each group

Group	n	Male/Female	Age (years)	DU/CG
OAC	33	20/13	43.5(18-70)	18/15
OFC	33	19/14	40.8(20-70)	17/16
OMC	33	21/12	41.6(19-69)	18/15
OFA	33	20/13	41.2(20-70)	19/14
Total	132	80/52	42.0(18-70)	72/64

### *H. pylori* eradication rates

*H. pylori* eradication rates were significantly different in patients receiving OAC and OFC than in those receiving OMC and OFA ( $P < 0.05$ ) (Tables 2 and 3). In the logistic regression model including treatment regimen, age, sex and endoscopes diagnosis, treatment regimens were identified as an independent factor responsible for the difference in the eradication rate (Table 3).

**Table 2** *H. pylori* eradication rate in each group

Group	n	Per protocol		Intent to treat		
		Eradication rate (%)	Confidence interval (95%)	n	Eradication rate (%)	Confidence interval (95%)
OAC	31	90.3	79.8-95.6	33	84.9	80.1-92.3
OFC	33	90.9	78.5-97.3	33	90.9	79.6-95.4
OMC	31	70.9	64.0-81.7	33	66.7	62.5-76.7
OFA	32	65.6	59.9-72.2	33	63.6	60.2-71.6
Total	127	79.5	72.4-82.5	33	76.5	70.5-81.8

**Table 3** *H. pylori* eradication rate in each group in relation to endoscopic diagnosis

Group	Duodenal ulcer		Chronic gastritis	
	n	Eradication rate (%)	n	Eradication rate (%)
OAC	17	88.2	14	92.9
OFC	17	94.1	16	87.5
OMC	16	68.8	15	73.3
OFA	19	57.9	13	76.9
Total	69	76.8	58	82.8

### Incidence of side effects

The incidence of side effects varied among the treatment regimens (Table 4). All of side effects were slight. A compliance of >90% was achieved for all the patients who completed the study.

**Table 4** Incidence of side effects in each group

Side effects	Incidence of side effects in each group patients (%)				
	OAC (n=31)	OFC (n=33)	OMC (n=31)	OFA (n=32)	Total (n=127)
Gastroenteric reactions	6.45	9.09	12.9	9.38	9.45
Skin eruption	6.45	0	3.23	6.25	3.94
Headache	6.45	6.06	3.23	0	4.72
Glossitis	0	0	3.23	0	0.79
Weakness	0	0	3.23	0	0.79
Fever	0	3.03	0	0	0.79
Somnolence	3.23	0	3.23	0	1.57

## DISCUSSION

In 1990, the 14-d bismuth triple therapy was recommended in the Ninth World Gastroenterology Conference in Sydney. Due to its high incidence of side effects (high than 30%) and poor compliance, this regimen has been replaced with other short-term 7-day triple therapy regimens that are more efficient and had fewer and milder side effects<sup>[13-19]</sup>. These new regimens include OMC 250 and OAC 500, which achieved *H. pylori* eradication rates of more than 90% in the MACH-1 study<sup>[20-26]</sup>. However, the eradication rates with those regimens decreased due to emergence of metronidazole resistance in *H. pylori* over the past few years. It has been reported that prevalence of metronidazole resistant *H. pylori* strains has increased to more than 70% in China and other countries<sup>[27-31]</sup>. This accounts for the failure of *H. pylori* eradication with metronidazole triple therapy.

With the wide application of anti-*H. pylori* therapy and antibiotic abuse, drug resistance in *H. pylori* has becomes an increasingly serious problem and a main reason of poor curative effect. At present<sup>[30,31]</sup>, the resistance to clarithromycin in *H. pylori* is diverse in the world. South-north difference existed such as the drugs used to treat other infection before (mainly respiratory infection). There are significant difference in the prevalence of metronidazole resistance between developed and developing countries. High prevalence of metronidazole resistance mainly relates to the wide application in parasite infection, dental infection and gynecological diseases in developing countries. Now there is a tendency that metronidazole resistance in *H. pylori* is increasing in the developed countries, probably due to the application of anti-*H. pylori* therapy. In spite of wide application of treatment with amoxicillin, amoxicillin resistance in *H. pylori* was rare.

In order to overcome the problem of metronidazole resistance and to compare the clinical efficacy of triple therapy regimens containing clarithromycin, we carried out this study. We achieved relatively high eradication rates for the clarithromycin-containing regimens OAC and OFC (90.3% and 90.9%, respectively). On the other hand, the eradication rate was relatively low for the metronidazole-containing regimen OMC and OFA. Taken together, we conclude that OAC and OFC are efficient regimens in eradicating *H. pylori* infection. Since the cost of furazolidone in OFC regimen is cheap and the *H. pylori* eradication rate of OFC regimen is high, we recommend that this regimen be one of choices for *H. pylori* eradication.

## REFERENCES

- 1 **Marzio L**, Cellini L, Angelucci D. Triple therapy for 7 days vs triple therapy for 7 days plus omeprazole for 21 days in treatment of active duodenal ulcer with *Helicobacter pylori* infection. A double blind placebo controlled trial. *Dig Liver Dis* 2003; **35**: 20-23

- 2 **Sargyn M**, Uygur-Bayramicli O, Sargyn H, Orbay E, Yavuzer D, Yayla A. Type 2 diabetes mellitus affects eradication rate of *Helicobacter pylori*. *World J Gastroenterol* 2003; **9**: 1126-1128
- 3 **Yamada T**, Miwa H, Fujino T, Hirai S, Yokoyama T, Sato N. Improvement of Gastric Atrophy After *Helicobacter pylori* Eradication Therapy. *J Clin Gastroenterol* 2003; **36**: 405-410
- 4 **Li S**, Lu AP, Zhang L, Li YD. Anti-*Helicobacter pylori* immunoglobulin G (IgG) and IgA antibody responses and the value of clinical presentations in diagnosis of *H pylori* infection in patients with precancerous lesions. *World J Gastroenterol* 2003; **9**: 755-758
- 5 **Konturek SJ**, Brzozowski T, Konturek PC, Kwiecien S, Karczewska E, Drozdowicz D, Stachura J, Hahn EG. *Helicobacter pylori* infection delays healing of ischaemia-reperfusion induced gastric ulcerations: new animal model for studying pathogenesis and therapy of *H pylori* infection. *Eur J Gastroenterol Hepatol* 2000; **12**: 1299-1313
- 6 **Goh KL**. Update on the management of *Helicobacter pylori* infection, including drug-resistant organisms. *J Gastroenterol Hepatol* 2002; **17**: 482-487
- 7 **Wolle K**, Leodolter A, Malfertheiner P, Konig W. Antibiotic susceptibility of *Helicobacter pylori* in Germany: stable primary resistance from 1995 to 2000. *J Med Microbiol* 2002; **51**: 705-709
- 8 **Bruley Des Varannes S**. How to treat after *Helicobacter pylori* eradication failure? *Gastroenterol Clin Biol* 2003; **27**: 478-483
- 9 **Hua JS**, Bow H, Zheng PY, Khay-Guan Y. Prevalence of primary *Helicobacter pylori* resistance to metronidazole and clarithromycin in Singapore. *World J Gastroenterol* 2000; **6**: 119-121
- 10 **Yeh YC**, Chang KC, Yang JC, Fang CT, Wang JT. Association of metronidazole resistance and natural competence in *Helicobacter pylori*. *Antimicrob Agents Chemother* 2002; **46**: 1564-1567
- 11 **Latham SR**, Labigne A, Jenks PJ. Production of the RdxA protein in metronidazole -susceptible and -resistant isolates of *Helicobacter pylori* cultured from treated mice. *J Antimicrob Chemother* 2002; **49**: 675-678
- 12 **Ivashkin VT**, Lapina TL, Bondarenko OY, Sklanskaya OA, Grigoriev PY, Vasiliev YV, Yakovenko EP, Gulyaev PV, Fedchenko VI. Azithromycin in a triple therapy for *H pylori* eradication in active duodenal ulcer. *World J Gastroenterol* 2002; **8**: 879-882
- 13 **Marais A**, Bilardi C, Cantet F, Mendz GL, Megraud F. Characterization of the genes rdxA and frxA involved in metronidazole resistance in *Helicobacter pylori*. *Res Microbiol* 2003; **154**: 137-144
- 14 **O' Morain C**, Borody T, Farley A, De Boer WA, Dallaire C, Schuman R, Piotrowski J, Fallone CA, Tytgat G, Megraud F, Spenard J. International multicentre study. Efficacy and safety of single-triple capsules of bismuth biskalcitrate, metronidazole and tetracycline, given with omeprazole, for the eradication of *Helicobacter pylori*: an international multicentre study. *Aliment Pharmacol Ther* 2003; **17**: 415-420
- 15 **Houben MH**, van de Beek D, Hensen EF, de Craen AJ, van 't Hoff BW, Tytgat GN. *Helicobacter pylori* eradication therapy in The Netherlands. *Scand J Gastroenterol Suppl* 1999; **230**: 17-22
- 16 **Wong BC**, Wang WH, Berg DE, Fung FM, Wong KW, Wong WM, Lai KC, Cho CH, Hui WM, Lam SK. High prevalence of mixed infections by *Helicobacter pylori* in Hong Kong: metronidazole sensitivity and overall genotype. *Aliment Pharmacol Ther* 2001; **15**: 493-503
- 17 **Qasim A**, O' Morain CA. Review article: treatment of *Helicobacter pylori* infection and factors influencing eradication. *Aliment Pharmacol Ther* 2002; **16**: 24-30
- 18 **Wong BC**, Wong WM, Yee YK, Hung WK, Yip AW, Szeto ML, Li KF, Lau P, Fung FM, Tong TS, Lai KC, Hu WH, Yuen MF, Hui CK, Lam SK. Rabeprazole-based 3-day and 7-day triple therapy vs omeprazole-based 7-day triple therapy for the treatment of *Helicobacter pylori* infection. *Aliment Pharmacol Ther* 2001; **15**: 1959-1965
- 19 **Choi JJ**, Jung HC, Choi KW, Kim JH, Ahn DS, Yang US, Rew JS, Lee SI, Rhee JC, Chung IS, Chung JM, Hong WS. Efficacy of low-dose clarithromycin triple therapy and tinidazole-containing triple therapy for *Helicobacter pylori* eradication. *Aliment Pharmacol Ther* 2002; **16**: 145-151
- 20 **Lind T**, Veldhuyzen van Zanten S, Unge P, Spiller R, Bayerdorffer E, O' Morain C, Bardhan KD, Bradette M, Chiba N, Wrangstadh M, Cederberg C, Idstrom JP. Eradication of *Helicobacter pylori* using one-week triple therapies combining omeprazole with two antimicrobials: the MACH I Study. *Helicobacter* 1996; **1**: 138-144
- 21 **Zanten SJ**, Bradette M, Farley A, Leddin D, Lind T, Unge P, Bayerdorffer E, Spiller RC, O' Morain C, Sipponen P, Wrangstadh M, Zeijlon L, Sinclair P. The DU-MACH study: eradication of *Helicobacter pylori* and ulcer healing in patients with acute duodenal ulcer using omeprazole based triple therapy. *Aliment Pharmacol Ther* 1999; **13**: 289-295
- 22 **Sung JJ**, Chan FK, Wu JC, Leung WK, Suen R, Ling TK, Lee YT, Cheng AF, Chung SC. One-week ranitidine bismuth citrate in combinations with metronidazole, amoxycillin and clarithromycin in the treatment of *Helicobacter pylori* infection: the RBC-MACH study. *Aliment Pharmacol Ther* 1999; **13**: 1079-1084
- 23 **Malfertheiner P**, Bayerdorffer E, Dietsch U, Gil J, Lind T, Misiuna P, O' Morain C, Sipponen P, Spiller RC, Stasiewicz J, Treichel H, Ujszaszy L, Unge P, Zanten SJ, Zeijlon L. The GU-MACH study: the effect of 1-week omeprazole triple therapy on *Helicobacter pylori* infection in patients with gastric ulcer. *Aliment Pharmacol Ther* 1999; **13**: 703-712
- 24 **Unge P**. The OAC and OMC options. *Eur J Gastroenterol Hepatol* 1999; **11**: 23-24
- 25 **Spiller RC**. Is there any difference in *Helicobacter pylori* eradication rates in patients with active peptic ulcer, inactive peptic ulcer and functional dyspepsia? *Eur J Gastroenterol Hepatol* 1999; **11**: 43-45
- 26 **Megraud F**, Lehn N, Lind T, Bayerdorffer E, O' Morain C, Spiller R, Unge P, van Zanten SV, Wrangstadh M, Burman CF. Antimicrobial susceptibility testing of *Helicobacter pylori* in a large multicenter trial: the MACH 2 study. *Antimicrob Agents Chemother* 1999; **43**: 2747-2752
- 27 **Jenks PJ**, Edwards DI. Metronidazole resistance in *Helicobacter pylori*. *Int J Antimicrob Agents* 2002; **19**: 1-7
- 28 **Calvet X**, Ducons J, Guardiola J, Tito L, Andreu V, Bory F, Guirao R. One-week triple vs quadruple therapy for *Helicobacter pylori* infection-a randomized trial. *Aliment Pharmacol Ther* 2002; **16**: 1261-1267
- 29 **Owen RJ**, Ferrus M, Gibson J. Amplified fragment length polymorphism genotyping of metronidazole-resistant *Helicobacter pylori* infecting dyspeptics in England. *Clin Microbiol Infect* 2001; **7**: 244-253
- 30 **Yakoob J**, Fan X, Hu G, Liu L, Zhang Z. Antibiotic susceptibility of *Helicobacter pylori* in the Chinese population. *J Gastroenterol Hepatol* 2001; **16**: 981-992
- 31 **Isakov V**, Domareva I, Koudryavtseva L, Maev I, Ganskaya Z. Furazolidone-based triple 'rescue therapy' vs quadruple 'rescue therapy' for the eradication of *Helicobacter pylori* resistant to metronidazole. *Aliment Pharmacol Ther* 2002; **16**: 1277-1282

Edited by Wang XL



# Pathobiological behavior and molecular mechanism of signet ring cell carcinoma and mucinous adenocarcinoma of the stomach: A comparative study

Xue-Fei Yang, Lin Yang, Xiao-Yun Mao, Dong-Ying Wu, Su-Min Zhang, Yan Xin

**Xue-Fei Yang, Xiao-Yun Mao, Dong-Ying Wu, Su-Min Zhang, Yan Xin**, The Fourth Laboratory, Cancer Institute, The First Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning Province, China

**Lin Yang**, Department of Pathology, Cancer Hospital, Chinese Academy of Medical Sciences, Beijing 100021, China

**Supported by** the National Natural Science Foundation of China, No.30070845 and No.30371607

**Correspondence to:** Professor Yan Xin, The Fourth Laboratory, Cancer Institute, The First Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning Province, China. yxin@mail.cmu.edu.cn

**Telephone:** +86-24-23256666 Ext 6351 **Fax:** +86-24-23253443

**Received:** 2003-09-09 **Accepted:** 2003-10-22

## Abstract

**AIM:** To elucidate the distinctive pathobiological behavior between signet ring cell carcinoma (SRC) and mucinous adenocarcinoma of the stomach.

**METHODS:** Based on the histological growth patterns and cell-functional differentiation classifications of stomach carcinoma, we conducted a series of comparative studies. All paraffin-embedded and frozen blocks were collected from the files of Cancer Institute of China Medical University. On the basis of histopathological observation, we applied enzymatic and mucous histochemistry, immunohistochemistry, flow cytometry (FCM) and molecular biology to compare these two categories of gastric cancers in terms of the DNA ploidy, proliferative kinetics, the expression of gastric carcinoma associated gene product and instabilities of mitochondrial DNA (mtDNA).

**RESULTS:** Gastric SRC was commonly seen in females below 45 years, mostly presenting diffuse growth and ovary or uterine cervix metastasis. The majority of SRC were absorptive and mucus-producing functional differentiation type (AMPFDT), which growth relied on estrogen. Meanwhile, stomach mucinous adenocarcinomas were mostly observed in males over 50 years, prone to massive growth or nest growth and extensive peritoneal infiltration, showing two categories of cell-functional differentiation types: AMPFDT and mucus-secreting functional differentiation type (MSFDT). Expressions of ER, enzyme c-PDE and 67kDaLN-R in SRC were evidently higher than that in mucinous adenocarcinoma, while expressions of LN, CN-IV, CD44v6, and PTEN protein were obviously lower in SRC than that in mucinous adenocarcinoma ( $P < 0.05$ ). There was no statistic significance in VEGF, ECD and instabilities of mtDNA ( $P > 0.05$ ) between the above two gastric carcinomas.

**CONCLUSION:** Though SRC and mucinous adenocarcinoma were both characterized by abundant mucus-secretion, they were quite different in morphology, ultrastructure, cell-functional differentiation and protein expression, indicating different mechanisms of carcinogenesis. We concluded that

combining histological growth patterns, cell-functional differentiation types with tumor related markers might be significant in early diagnosis and prognosis assessment for SRC and mucinous adenocarcinoma of the stomach.

Yang XF, Yang L, Mao XY, Wu DY, Zhang SM, Xin Y. Pathobiological behavior and molecular mechanism of signet ring cell carcinoma and mucinous adenocarcinoma of the stomach: A comparative study. *World J Gastroenterol* 2004; 10(5): 750-754

<http://www.wjgnet.com/1007-9327/10/750.asp>

## INTRODUCTION

SRC and mucinous adenocarcinoma of the stomach were generally confounded as “mucoïd carcinoma” until 1964 when Zhang *et al* proposed that the so-called “mucoïd carcinoma” included two categories that presented different growth patterns. “Mucoïd carcinoma” was finally divided into mucinous adenocarcinoma and SRC in WHO’s histological classification of the stomach in 1974. At that time, it remained unclear of the different biological behaviors and metastatic or infiltrative characteristics of these two stomach carcinomas.

From 1962, Cancer Institute of China Medical University began a series of studies on histological growth patterns, cell-functional phenotype classifications and infiltrative, metastatic patterns of stomach carcinoma, and a series of comparative studies on SRC and mucinous adenocarcinoma of the stomach were also carried out the following 40 years. We classified functional differentiation types of gastric cancer by histopathological observation, enzymatic and mucous histochemistry, and detected the expressions of related genes with the help of biological techniques. Different biological behaviors of local infiltration and metastasis of SRC and mucinous adenocarcinoma were investigated at morphological, functional and protein levels.

## MATERIALS AND METHODS

All paraffin-embedded and frozen blocks were collected from the files of Cancer Institute of China Medical University. On the basis of histopathological observation, AKP, ACP and LAP were detected by enzymatic histochemistry and various kinds of mucin were detected by mucous histochemistry in order to propose a new cell-functional classification of gastric carcinomas. We observed the expression of ER, cPDE, laminin (LN) and its receptor 67kDaLN-R, collagen-IV (CN-IV), CD44v6, VEGF, ECD and PTEN proteins by immunohistochemistry, the variation of DNA ploidy, proliferative kinetics by flow cytometry (FCM), and three adjacent regions of mtDNA (D-loop, tRNA<sup>phe</sup> and 12S rRNA) were detected for instabilities via PCR amplification followed by direct DNA sequencing and dHPLC. All procedures were done according to references<sup>[1-11]</sup>.

## RESULTS

SRC and mucinous adenocarcinoma were found quite different

in pathological morphology, functional differentiation phenotypes, molecular pathology mechanisms and prognosis as follows.

### Morphologic observation

The different growth patterns of mucinous adenocarcinoma and SRC were firstly proposed, following the histological growth classifications of the stomach in 1964. The former presented nest or massive growth, which cancer cells produced and secreted abundant mucus-like substance and then hoarded in the cancerous nest; while the latter presented signet ring cells, widely infiltrated in the stomach wall, accompanied by the formation of “migratory cancerous embolus of lymphatic vessel”. SRC had specific cytogenetics: cancer cells were separate, and presented irregular amebocyte shape. Image of ameboidism was often observed emigrating from lymphatic vessel especially in loose part of the stomach (subserosa and submucosa), which suggested the characteristic of widespread growth and invasion. This image was not shown in mucinous adenocarcinoma of nest or massive growth. Meanwhile, the mesenchymal reaction was also quite different between the above two stomach carcinomas: cancerous foci of mucinous adenocarcinoma was mainly enwrapped by collagen fibers, and the argyrophilia fibers were thick and intensive to bundles, surrounding the outside of the cancerous foci; some transition to collagen fibers and even basement membrane-like structures were shown. There were such lymphoid cells and macrophages as inflammatory reaction in adjacent cancerous foci. However, mesenchyma of SRC was loose and in edema, and argyrophilia fibers were reticularly loose, which suggested that the host

immunological reaction was weaker than that of the mucinous adenocarcinoma and coincident with its diffusely infiltrating growth pattern.

### Functional differentiation vs invasion and metastatic characteristics

Functional differentiation investigation showed that more than 80.0% of SRC showed AMPFDT: SRC cells not only produced mucus, but also expressed intestinal enzymes of absorption cell markers (AKP and LAP), ACP, and estrogen receptor (ER) in most cancer cells.

Mucinous adenocarcinoma showed specific functional differentiation: 55.3%(21/38) of them showed single MSFDT; 44.7%(17/38) were AMPFDT. The latter was similar to SRC in biological characteristics, while the former did not express intestinal enzymes of absorption cell markers or ER (Table 1).

### Tumor related markers

Expressions of ER, enzyme c-PDE and 67kuN-R were evidently higher in SRC than in mucinous adenocarcinoma, while expressions of LN, CN-IV, CD44v6, and PTEN protein were obviously lower in SRC than those in mucinous adenocarcinoma ( $P<0.05$ ). There was no statistic significance in VEGF, ECD and instabilities of mtDNA ( $P>0.05$ ) between the two gastric carcinomas. In SRC, DNA ploidy was different at different pathologic stages: the elevated contents of DNA, polyploid and heteroploid were often seen in early SRC; while the content of DNA was lower and diploid or subdiploid were often presented in advanced SRC. Polyploid and heteroploid were often observed in mucinous adenocarcinoma (Table 1).

**Table 1** The comparison of pathobiologic characteristics between SRC and mucinous adenocarcinoma

Pathobiologic characteristics	SRC (AMPFDT)	Mucinous adenocarcinoma	
		AMPFDT	MSFDT
Onset age	Mostly below 50	Same as the left	Mostly over 50 <sup>[1,2]</sup>
Sex	more in female	Same as the left	More in male <sup>[1,2]</sup>
Infiltrative depth	16.7% (2/12) passing through the serosa		70.0% (7/10) passing through the serosa <sup>[1,2]</sup>
Growth pattern	Diffuse growth 100.0%(12/12)		Diffuse growth 40.0%(4/10), massive growth 60.0%(6/10) <sup>[1,2]</sup>
Metastasis rate of lymph node	66.7% (8/12)		80.0%(8/10) <sup>[1,2]</sup>
Distant metastasis	Often to ovary and uterus, sometimes to bones, marrow, bladder, blepharon or skin of lower extremities	Same as the left	Prone to peritoneal extensive infiltration <sup>[1,2]</sup>
Tumor related markers			
ER	Positive rate 75.0%(9/12)		Positive rate 10.0%(1/10) <sup>[1,2]</sup>
c-PDE	Positive rate 80.0%(4/5)		Positive rate 20.0%(1/5) <sup>[3]</sup>
LN	Few, positive rate 25.0%(2/8)		BM*-like line structure was seen frequently, positive rate 83.3%(5/6) <sup>[4]</sup>
67KDa LN-R	Positive rate 75.0%(15/20)		Positive rate 25.0%(1/4) <sup>[5]</sup>
Collagen IV	CN-IV positive fragments or particles only, positive rate 87.5%(7/8)		BM*-like line structure was seen frequently
CD44V <sub>6</sub>	Positive rate 11.8%(2/17)		Positive rate 100.0%(6/6) <sup>[4]</sup>
PTEN	Low expression (25.0%, 7/21)		Positive rate 62.5%(5/8) <sup>[6]</sup>
VEGF	Positive rate 90.0%(9/10)		High expression (60.0%) 6/10 <sup>[8]</sup>
ECD	Negative expression 85.0% (17/20)		Positive rate 100.0%(7/7) <sup>[7]</sup>
DNA ploidy	Negative expression 85.0% (17/20)		Negative rate 75.0%(3/4) <sup>[9]</sup>
	Polyploid and heteroploid were often seen in early stage, while diploid and hypodiploid were often seen in advanced stage		Polyploid and heteroploid were often observed <sup>[10]</sup>
mtDNA variation	66.7%(2/3), para-cancerous		66.7%(2/3), para-cancerous
D-loop region	tissue 33.3%(1/3)		tissue 66.7%(2/3)
12S rRNA	33.3%(1/3)		66.7%(2/3) <sup>[11]</sup>
Ultrastructure	More mucous secretion. Few desmosome junctions and gap junctions between cells.		Plenty of mucous secretion. Several desmosome junctions and gap junctions between cells
5 year survival rate	15.9%		19.4%

\*Basement membrane.

## DISCUSSION

The survival rate of both SRC and mucinous adenocarcinoma was extremely low. According to the National Cooperation Group of Stomach Carcinoma, the 5-year survival rate of SRC and mucinous adenocarcinoma of the stomach was only 15.9% and 19.4% respectively mainly because most clinical cases of SRC or mucinous adenocarcinomas were moderate to advanced cancer accompanied with extensive infiltration and metastasis. Metastasis, especially unmanageable metastasis to the remote important viscera was the primary cause of patients' death. Research findings of more than 40 years revealed different infiltrative and metastatic mechanisms of SRC and mucinous adenocarcinoma at morphological, functional and protein levels, based on histological growth patterns and cell functional differentiation phenotypes.

The structural basis of different growth patterns of SRC and mucinous adenocarcinoma was demonstrated by electron microscopic observation and mucus histochemistry by Wang *et al* (Cell Biology Institute of China Medical University) and other Chinese scholars<sup>[12]</sup>. Under electron microscope, SRC was lack of free ribosomes but rich in rough endoplasmic reticulum (RER), lysosomes, mucus granules, and Golgi complex presented cystiform dilatation, which suggested SRC cells had a strong capability of protein and mucus synthesis. In addition, there were few microvilli on the surface of SRC cell membrane, desmosome junction and gap junction ultimately vanished, the interspaces of cancer cells were enlarged, which suggested that adhesive ability of cancer cells decreased and detachment was easy among cells. Histochemical observation found that these changes were accompanied by releasing of multiple sialomucin, sulfuric acid mucopolysaccharide (AMP), and polysaccharide hydrolases such as acid phosphatase (ACP). These characteristics contributed to the potential of dissolving surrounding tissues, and strong infiltration and metastasis. Nevertheless, cancer cells of mucinous adenocarcinoma were rich in free ribosomes, with scattered RER, and fewer Golgi complex and lysosomes than that of SRC. Further, different from SRC, microvilli and part of desmosome junction and gap junction were shown on the surface of cell membrane. All above characteristics suggested mucinous adenocarcinoma had a stronger adhesive ability than SRC, which partly explained their different growth patterns.

In 1979, Zhang *et al* firstly reported that diffusely growing SRC could show retrograde metastasis from the stomach to cervix and parametrium via "migratory cancerous embolus"<sup>[13]</sup>, which raised two questions at the same time: Why was it often seen that SRC or poorly differentiated adenocarcinoma and undifferentiated carcinoma containing signet ring cell metastasized to cervix and parametrium, but it was rarely seen in mucinous adenocarcinoma? Was there any difference in cell-functional differentiation and associated molecular pathological characteristics apart from morphologic differences?

The authors detected cell-functional differentiation of gastric cancer cells by enzymatic, mucus histochemistry and immunohistochemistry, proposed a new concept of cell-functional classification of stomach carcinoma, and found that the carcinoma of different functional types were quite different in local infiltration and metastasis to remote viscera<sup>[1,2]</sup>. Based on this study, we extensively investigated the hypostases of different biological behaviors of the above two carcinomas<sup>[2]</sup>. According to cell-functional differentiation of cancer cells, more than 80.0% of SRC showed AMPFDT, which suggested it was a specific carcinoma cell type with disturbed cell-functional differentiation, possessing both absorptive and mucus-producing functions, and its growth and infiltration relied on ER. SRC not only produced mucus, but also expressed small intestine absorptive cell marker enzymes and ACP. Mucus-producing functional differentiation and the release

of ACP in SRC contributed to hydrolyze mesenchyma surrounding the cancerous foci and invade into normal tissue; and absorptive functional differentiation contributed to absorb nutrition from the host, which accelerated its malignant proliferation. SRC cells customarily expressed ER, which suggested that its growth relied on the existence of estrogen. The reason why SRC was often observed in the female of the premenopause and prone to metastasize to ovary or uterine cervix was its high affinity to organs with a high level of estrogen such as ovary. During the metastasis from the stomach to ovary, "ER (on cancer cells)-estrogen (in the ovary)" affinity linkage might play an important role. Our results suggested that under the prerequisite of hematogenous metastasis, lymphatic metastasis or implantation metastasis to the ovary or cervix, cancer cells with high activities of ER had specific affinity and adaptability to organs rich in estrogen and thus could easily metastasize to the ovary or cervix<sup>[2]</sup>.

Mucinous adenocarcinoma exhibited two functional classifications as AMPFDT and MSFDT. The biological behaviors and part of molecular pathological characteristics of the former were similar to that of SRC; but the latter did not express small intestine absorptive cell marker enzymes, which disabled its absorptive activity, decreased absorption of enough nutrition and restrained the growth and spread. It was probably one reason why mucinous adenocarcinoma was prone to massive or nest growth, and thus exhibited better prognosis than SRC. These two functional classifications of mucinous adenocarcinoma were quite different in onset age, serosa infiltration, dependence on estrogen (ER expression), *etc.* It was suggested that mucinous adenocarcinoma had the heterogeneity both in cell-functional differentiation and biological feature. So special attention should be paid to cell-functional differentiation characteristics in judging malignant biological behaviors and metastatic patterns of mucinous adenocarcinoma.

In order to explore the difference of molecular mechanisms between the above two gastric carcinomas, we also designed a series of molecular pathological markers ranging from cell proliferation and differentiation, extracellular matrix, tumor angiogenesis, tumor suppressor genes, *ect.* Cyclic nucleotide acid phosphodiesterase (cPDE) is a key enzyme degrading cyclic adenosine phosphate (cAMP) and cyclic guanosine phosphate (cGMP), thereby influencing cell proliferation and differentiation. This study revealed that SRC exhibited higher activities of cAMP-PDE and cGMP-PDE than mucinous adenocarcinoma, and it was concluded that SRC cells had stronger abilities of synthesizing and secreting cPDE than mucinous adenocarcinoma. And cAMP-PDE was a reliable enzymatic marker to estimate malignant degree and prognosis of the above two gastric carcinomas<sup>[3]</sup>. LN was the main ingredient of basement membrane. The studies found stomach cancer cells could not only destroy basement membrane but also unceasingly synthesize the ingredient of its basement membrane. Mucinous adenocarcinoma cells had the capacity of synthesizing, and excreting LN out of the cells, which formed a line-like structure similar to basement membrane. This might be one of the reasons why mucinous adenocarcinoma often grew in a nest or massive shape. SRC synthesized very few LN and there were only a few LN particles in extracellular matrix. This might be related to some characteristics of SRC, such as infiltrative growth and high invasiveness<sup>[4]</sup>. The quantity and expression intensity of 67KDa LN-R influenced the infiltrative migration of malignant tumor cells. Acting as a kind of LN receptor, 67KDa LN-R usually was involved in the identification of ECM and information convection. Increased expression of 67KDa LN-R in tumor cells would benefit adhesiveness and infiltration. Among the different histological types of gastric cancer, the expression of 67KDa LN-R was

highest in SRC (75.0%, 15/20), and lowest in mucinous adenocarcinoma (25.0%, 1/4), which might be in relation to their different abilities of infiltration and metastasis<sup>[5]</sup>. The change of LN expression level was closely correlated to the pathological behaviors of gastric cancer, and could be used as an objective marker to assess the growth ability and the tendency of hematogenous metastasis between the above two gastric cancers. CN-IV was expressed in the above two gastric cancers, but the expression pattern was quite different<sup>[4]</sup>. In mucinous adenocarcinoma, the basement membrane-like structure containing CN-IV was seen clearly. However, only linear fragments or positive particles containing CN-IV were seen in SRC. The findings suggested that the above two gastric cancers had different capacities of synthesizing and secreting CN-IV hydrolase. CN-IV hydrolase was strong in the interstitial of SRC, and hydrolyzed CN-IV into discontinuous fragments or particles, which led to the infiltrative growth of cancer cells. The expression of CN-IV in gastric cancer presented negative correlation with its infiltrative growth ability, and could be used as one of the markers to evaluate the poor prognosis of the above two gastric cancers<sup>[4]</sup>.

CD44v6 took part in specific adhesion processes between cells or cells and matrix. Our results<sup>[6,14]</sup> showed CD44v6 had a close relation to metastatic potential of gastric cancer cells and the poor prognosis of patients. It was found that the protein expression of CD44v6 was quite higher in mucinous adenocarcinoma than in SRC. This was probably due to the crypt formation and nest or massive growth pattern of mucinous adenocarcinoma. VEGF is correlated with tumor angiogenesis. A lot of evidence showed that VEGF greatly increased the probability of tumor metastasis by accelerating blood vessel growth. But between the above two gastric cancers, the difference of VEGF expression was not significant<sup>[7]</sup>. Up to now, PTEN/MMAC1/TEP1 gene is the first tumor suppressor gene that was proved to have phosphatase activity. Using immunohistochemistry, we found the expression of PTEN protein in SRC was significantly lower than in mucinous adenocarcinoma. The expression of PTEN protein presented negative correlation with gastric cancer's pathological grade and prognosis, which indicated that PTEN gene mutation or inactivation might be one of the reasons for the poorer prognosis of SRC compared with mucinous adenocarcinoma<sup>[8]</sup>. ECD is a kind of transmembrane glycoprotein, which induced the adhesion of homogenous cells. The dysfunction of ECD made the adhesion of homogenous cells impossible. Thus, cancer cells tended to separate from each other and metastasize. ECD presented negative expression in 85% (17/20) of SRC and 75% (3/4) of mucinous adenocarcinoma. So ECD could be used as one of the markers to evaluate the poor prognosis of the above two gastric cancers<sup>[9]</sup>.

There were some studies on tumor related markers from other aspects about the above two gastric cancers. For example, the expression of GST- $\pi$  was lower in SRC (50.0%, 4/8) than that in mucinous adenocarcinoma (66.7%, 6/9), and it was related to differentiation degree of cancer cells. The expression of GST- $\pi$  in well differentiated cells was higher, and it was closely correlated with carcinogenesis and progress of gastric cancer, but there was no significance<sup>[15]</sup>. S-Tn antigen is a prosoma of saliva acidulated T antigen, and belongs to the embryo or differentiated antigen. It might take part in the early malignant transformation of gastric cancer. It has been reported that the expression of S-Tn antigen in SRC was highest among different histological types of gastric cancers<sup>[16]</sup>, while the mechanism is still unknown.

DNA aneuploidy of tumor cells is one of its malignant characteristics. DNA aneuploidy of gastric cancer is closely related to the remote organic metastasis, especially to the hematogenous metastasis<sup>[10]</sup>. There were a lot of polyploid and

heteroploid cells in early SRC, while most of advanced SRC showed diploid or hypodiploid cells. It suggested that the reduction of DNA content in advanced SRC cells was probably related to the accumulation and crushing of mucus. SRC had the tendency to infiltrate into submucosa in early stage, and this behavior was related to the DNA heteroploidy of cancer cells. Detection of DNA ploidy, especially early detection would help to estimate the malignant level and poor prognosis of the above two gastric cancers.

Recently, researchers have paid more attention to the relationship between the damage of mtDNA and carcinogenesis. mtDNA is a 16 569-bp double stranded, closed circular molecule, which encodes polypeptides participating in oxidative phosphorylation and synthesis of ATP. Compared to nuclear DNA, mtDNA is more susceptible to damage, because of the lack of protective action by histones and the limited capacity of damage repairing system as in yeast and most nuclear genomes. Mitochondrial D-loop and hypervariable region suffered the attack of ROS easily. Chomyn *et al* considered that mtDNA was not necessary to the start-up of necrosis, but it influenced the speed of necrosis because the decrease or damage of mtDNA would result in the increase of ROS<sup>[17]</sup>. Our study found that there was no statistic significance in instabilities in D-loop region between gastric cancerous and para-cancerous tissues. There was a significant correlation between differentiation degree of gastric cancer and variant frequencies of 12S rRNA-tRNA<sup>phe</sup>. The poorly differentiated gastric cancers were more prone to 12S rRNA-tRNA<sup>phe</sup> variations. But there was no significance between SRC and mucinous adenocarcinoma<sup>[11]</sup>. This result needs to be proved by larger sample studies. The relationship between mtDNA and gastric cancer would further be clarified.

There were several molecular biology studies about gastric SRC and mucinous adenocarcinoma. MSI is a frequent genetic change in gastric cancer. Up to now, the average rate of MSI was 33.9% among 29 sites that have been detected, and phenotypes of MSI were different in gastric cancers due to their different pathological types<sup>[18,19]</sup>. MSI-positive frequency of SRC was significantly higher than mucinous adenocarcinoma of the stomach, which indicated that the occurrence and progress of the above two gastric cancers probably had different molecular mechanisms. Loss of heterozygous (LOH) is one of MSI phenotypes. The fractional allelic loss (FAL) (the odds of LOH positive marker number and MSI marker number) was related to infiltrative ability of gastric cancer cells and different growth patterns of gastric cancer. Our studies suggested that there might be different allelic deletion between SRC and mucinous adenocarcinoma, which might clarify that the different heredity phenotypes and biological behaviors of the above two gastric cancers were closely correlated with MSI and LOH. We plan on marking MSI and screening LOH site so as to discover minimal deletion fragment nearby these sites by molecular biology and LCM technique. This would lead us to detect some unknown TSGs and study the molecular biological mechanisms of different biological behaviors of the above two gastric cancers.

In conclusion, from the view of histological growth patterns and cell-functional differentiation types of gastric cancer, we investigated various kinds of pathobiological behaviors of gastric SRC and mucinous adenocarcinoma. On the basis of this, we studied on tumor related markers for early diagnosis and prognosis evaluation of gastric cancer, so to instruct clinical surgical treatment scientifically and effectively, and improve survival rate and survival quality. In our cancer institute, the 5 year survival rate of gastric cancer has risen from 19.6% to 58.5%, approaching international advanced level<sup>[20]</sup>. Along with the preliminary accomplishment of human genome project, the studies on tumor genomics are advancing with

every passing day. A lot of tumor related genes will be found, and their roles need to be clarified in occurrence, progress and prognosis of the above two gastric cancers. Meanwhile, little literature has been reported on the morphogenetic or histogenetic mechanisms and early diagnostic markers of gastric SRC and mucinous adenocarcinoma, and there is still much dispute in this field. All these problems need further research.

## REFERENCES

- 1 **Xin Y**, Li XL, Wang YP, Zhang SM, Zheng HC, Wu DY, Zhang YC. Relationship between phenotypes of cell-function differentiation and pathobiological behavior of gastric carcinomas. *World J Gastroenterol* 2001; **7**: 53-59
- 2 **Xin Y**, Zhao FK, Wu DY, Wang YP, Zhang YC. Comparative study of the pathobiological behavior in mucinous adenocarcinoma and signet ring cell carcinoma of the stomach. *Zhongguo Yike Daxue Xuebao* 1996; **25**: 441-443
- 3 **Xin Y**, Zhao FK, Wu DY, Zhang YC. Comparative study of cyclic nucleotide phosphodiesterases and marker enzymes of small intestinal absorptive cell in gastric cancer tissue. *Zhongguo Yike Daxue Xuebao* 1991; **20**: 333-337
- 4 **Zhao FK**, Zhang SM, Wang YP, Xu L, Wu DY, Xin Y. Relationship between the expression of collagen IV and laminin and the pathobiological behavior of gastric carcinomas. *Zhongguo Yike Daxue Xuebao* 1998; **27**(Suppl): 6-8
- 5 **Li XL**, Wang YP, Wu DY, Zhang SM, Xin Y. Expression of PTEN-encoding product, ECD and 67KDa laminin receptor and invasion and metastasis of gastric carcinomas. *Zhonghua Yixue Zazhi* 2003; **83**: 599-601
- 6 **Xin Y**, Zhao FK, Zhang SM, Wu DY, Wang YP, Xu L. Relationship between CD44v6 expression and prognosis in gastric carcinoma patients. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 210-214
- 7 **Wang YP**, Chen Y, Yang L, Wu DY, Zhang SM, Kuang LG, Sun HW, Zheng HC, Xin Y. Expression of VEGF in gastric carcinoma and its clinical significance. *Zhongguo Yike Daxue Xuebao* 2003; **32**: 184-186
- 8 **Zheng HC**, Chen Y, Kuang LG, Yang L, Li JY, Wu DY, Zhang SM, Xin Y. Expression of PTEN-encoding product in different stages of carcinogenesis and progression of gastric carcinoma. *Zhonghua Zhongliu Zazhi* 2003; **25**: 13-16
- 9 **Li XL**, Yang XF, Wu DY, Zhang SM, Xin Y. ECD and expression of PTEN gene an invasion and metastasis of gastric carcinomas. *Zhongguo Zhongliu Linchuang* 2003; **30**: 349-352
- 10 **Xu L**, Zhang SM, Wang YP, Zhao FK, Wu DY, Xin Y. Relationship between DNA ploidy, expression of ki-67 antigen and gastric cancer metastasis. *World J Gastroenterol* 1999; **5**: 10-11
- 11 **Han CB**, Li F, Zhao YJ, Ma JM, Wu DY, Zhang YK, Xin Y. Variations of mitochondrial D-loop region plus downstream gene 1 2S rRNA-tRNA<sup>phe</sup> and gastric carcinomas. *World J Gastroenterol* 2003; **9**: 1925-1929
- 12 **Zhu SY**, Tang XP, Zhang PY. Ultrastructure observation of angiogenesis and microvessel density in the signet ring cell carcinoma of stomach. *Dianzi Xianwei Xuebao* 2002; **21**: 900-902
- 13 **Zhang YC**, Zhang PF, Wei YH. Metastatic carcinoma of the cervix uteri from the gastrointestinal tract. *Gynecol Oncol* 1983; **15**: 287-290
- 14 **Xin Y**, Wang YP, Zhang SM, Wu DY, Xu L, Zhang YC. Relationship between CD44v6 expression and metastatic potential and prognosis in gastric carcinoma. *Chin Med Sci J* 2000; **15**: 128
- 15 **Lin LX**, Jiang HC, Zhu AL, Qi SY. The expression of GST- $\pi$  and P-gp in gastric carcinoma. *Zhongguo Zhongliu Linchuang Yu Kangfu* 2000; **7**: 23-24
- 16 **Qiao SX**, Wang WH, Yuan M, Tan SQ. The expression of S-Tn antigen in the tissues of gastric cancer and precancerous lesions. *Zhonghua Putong Waikexue Zazhi* 2000; **15**: 358-360
- 17 **Chomyn A**, Attardi G. mtDNA mutations in aging and apoptosis. *Biochem Biophys Res Commun* 2003; **304**: 519-529
- 18 **Kim KM**, Kwon MS, Hong SJ, Min KO, Seo EJ, Lee KY, Choi SW, Rhyu MG. Genetic classification of intestinal-type and diffuse-type gastric cancers based on chromosomal loss and microsatellite instability. *Virchows Arch* 2003; **443**: 491-500
- 19 **Wang Y**, Ke Y, Ning T, Feng L, Lu G, Liu W, E Z. Studies of microsatellite instability in Chinese gastric cancer tissues. *Zhonghua Yixue Yichuanxue Zazhi* 1998; **15**: 155-157
- 20 **Zhang WF**, Zhang YC, Chen JQ. *Gastric Carcinoma*. 2nd edition. Shanghai: Shanghai Kexue Jishu Chubanshe 2001: 264-265

Edited by Zhu LH Proofread by Xu FM

# Preventive effect of Ganfujian granule on experimental hepatocarcinoma in rats

Yan Qian, Chang-Quan Ling

**Yan Qian**, Department of Traditional Chinese Medicine, General Hospital of PLA, Beijing 100853, China

**Chang-Quan Ling**, Department of Traditional Chinese Medicine, Changhai Hospital, Second Military Medical University, Shanghai 200433, China

**Correspondence to:** Yan Qian, Department of Traditional Chinese Medicine, General Hospital of PLA, Beijing 100853, China. qianxden@yahoo.com

**Telephone:** +86-10-66937093

**Received:** 2003-06-21 **Accepted:** 2003-08-18

## Abstract

**AIM:** To investigate the inhibitory effect of dietary and medicinal formula Ganfujian granule on diethylnitrosamine (DEN)-induced hepatocarcinoma in rats.

**METHODS:** Male SD rats had free access to water containing 0.1 g/L DEN for 16 weeks, during which the rats fed with standard diet or administration of Ganfujian granule (30.4 g/Kg in diet). At weeks 4, 8, 12 and 16 of hepatocarcinogenesis 5 rats of each group were sacrificed, and at week 20 another 30 rats were sacrificed from each group. The end point for survival observation was at week 28. Immunohistochemistry methods were used to examine the effect of Ganfujian granule on the process of hepatocarcinogenesis including proliferation of hepatocytes and cell cycle modulation.

**RESULTS:** Ganfujian granule could reduce and delay the incidence of hepatocarcinoma in rats and prolong the survival of animals. In addition, Ganfujian granule had a marked inhibitory effect on high expression of cyclin dependent kinase (CDK4) during the whole process of hepatocarcinogenesis and cyclin D1 at week 16 and the number of proliferating cell nuclear antigen (PCNA) positive cells in different stages of hepatocarcinogenesis.

**CONCLUSION:** Ganfujian granule can reduce and delay the incidence of hepatocarcinoma in rats by exerting direct or indirect effects on cell cycle and inhibiting uncontrolled proliferation of hepatocytes.

Qian Y, Ling CQ. Preventive effect of Ganfujian granule on experimental hepatocarcinoma in rats. *World J Gastroenterol* 2004; 10(5): 755-757

<http://www.wjgnet.com/1007-9327/10/755.asp>

## INTRODUCTION

Liver cancer is one of the common malignancies in the world, especially in Asia and Africa. Despite many advances in the treatment of this disease in recent decades, its long-term therapeutic outcome remains poor and prognosis is devastating. Prevention seems to be the best strategy in lowering the present prevalence of the disease. Much work has been done in the prevention of liver cancer, including monitoring of high risk populations (residents in areas of high incidence, cirrhosis of

various causes and patients with chronic hepatitis)<sup>[1,2]</sup>, extensive immunization to prevent HBV infection<sup>[3]</sup>, and use of interferon to alleviate HCV infection, both providing hope of lowering or delaying the complication of hepatic cirrhosis or progression to liver cancer in chronic hepatitis patients<sup>[4]</sup>. More and more efforts have been made in search of natural materials and foods as a means of chemical prevention of liver cancer<sup>[5]</sup>. Characterized by low toxicity and effectiveness, traditional Chinese herbs have aroused more interest in the prevention of tumors<sup>[6]</sup>. Ganfujian granule is an oral granule consisting of dietary and medicinal Chinese herbs for preventing liver cancer, developed by our laboratory based on the basic traditional Chinese medicine (TCM) theories of “invigorating spleen and soothing liver” as the key principle in combination with our clinical experience in preventing and treating liver cancer. Our previous experiments have demonstrated that Ganfujian granule can effectively prevent the formation of liver preneoplastic lesions<sup>[7]</sup>. In the present study we attempted to observe the effect of Ganfujian granule on DEN induced liver cancer in rats and to explore the potential preventive mechanism from the perspective of modulation of proliferation of hepatocytes.

## MATERIALS AND METHODS

### Materials

Male SD rats (160-180 g in body mass) were obtained from the Animal Center of the Second Military Medical University of Shanghai. DEN and 2-bromo-3'-deoxyuridine (BrdU) marker were from Sigma, CDK4 sheep monoclonal antibody and cyclin D1 monoclonal antibody were from Santa Cruz, PCNA and BrdU test kit from DAKO.

### Methods

**Animal model** The rats had free access to food and water throughout the study. The basal diet was a standard diet. The Ganfujian granule-containing diets were prepared by mixing 3.04% Ganfujian granule with the standard diet (the Ganfujian granule amount for the rats was converted from the kg body mass daily for adults). After a 5 d acclimatization period, 165 rats were assigned into group I (carcinogen-exposed control) and group II (Ganfujian granule treatment). Fresh sterile water was used to prepare DEN solution of 100 µg/mL concentration, to which the rats had free access. The rats in group I were fed with basal diet and those in group II with Ganfujian granule containing diet. At wk 16 DEN water was discontinued and the food for the animals in group II was changed into basal diet. Five rats from each group were sacrificed at wk 4, 8, 12 and 16 of DEN hepatocarcinogenesis. Livers of the rats were resected, cut into 3 mm slices and fixed. Another 30 rats from each group were sacrificed at wk 20 to observe incidence rate of liver cancer. The remaining animals were raised continuously to observe survival. The end point of observation was at wk 28.

**Immunohistochemical staining** The liver tissues were fixed in 40g/L buffered formaldehyde and processed for embedding in paraffin, cut into 4 µm thick slices, deparaffinized with

xylene and washed with alcohol, and then processed for immunohistochemistry by using a streptavidin-biotin-peroxidase complex methods. Cyclin D1 rat monoclonal antibody, CDK4 rabbit polyclonal antibody and PCNA rat monoclonal antibody were used as primary antibodies. As controls, known positive tissue sections were used, and for negative controls exposure to the primary antibody was omitted.

To determine labeling index (LI), 5 rats from each group at wk 20 received intraperitoneal injections of 2-bromo-3'-deoxyuridine (BrdU) 35 mg/kg body mass 1 h before death. BrdU incorporation into nuclei was determined immunohistochemically with a 'BrdU test kit'.

### Analysis and quantification of staining results

Staining of cyclin D1 and CDK4 foci in liver tissue section was analyzed and quantified using an image analyzer (HPIAS-1000 highly clear color pathological graph analysis system). With the gray scale unified, five different fields were selected for each section to conduct automatic measurement by selecting the mean gray scale as the index of measurement,  $\times 400$ . The system uses 256 as the maximal gray of white and 0 as the minimal gray of black. The smaller the mean gray scale, the higher the positive reaction. The brownish yellow particles and dark blue particles covering the nuclei represented the positive signal of PCNA and BrdU respectively. The number of positive liver cells was counted. Ten un-overlapped high power fields ( $\times 40$ ) of the same transmigration were used for counting the positive and negative cells (100 cells).

### Statistical analysis

Data relative to tumor incidence were analyzed by Fisher's exact test. Survival functions were analyzed by the log-rank analysis. The differences between groups were analyzed by Student's *t* test, and the two-tailed statistical significance was determined. The statistical calculations were carried out by SPSS and NIOS software packages.

## RESULTS

### General effects of ganfujian granule

The liver surface of rats in the group I grew coarse gradually between 4-8 wk of DEN hepatocarcinogenesis, on which small granules of local focus were seen. Light microscopy showed that liver cells became degenerative and locally necrotic. Cirrhosis was evidently seen in 40% at wk 12, and at wk 16, cirrhosis was seen in all the rats (100%). More and larger nodules were seen on the liver surface, the liver became smaller, and deranged pseudobulbi of different sizes were seen histologically. Early cancer change was seen in 20% rats. At wk 20, all the 30 rats (100%) developed hepatocarcinoma. Liver impairment of group II was milder than that of group I at various stages of hepatocarcinogenesis. No cirrhosis was seen at wk 12, 60% developed cirrhosis at wk 16, but no cancerous change was observed, and at wk 20, 24 of the 30 rats (80%) developed liver cancer. Fisher's exact test analysis showed that the incidence rate of liver cancer between the two groups was significant ( $P < 0.05$ ). Using 28 wk as the end point of observation, the longest survival of rats in group I was 20 wk, and 28 wk in group II. Log-rank test showed that survival of rats in group II was longer than rats in group I, and risk of death in group I was lower than that in group II ( $P < 0.05$ , Figure 1).

### Changes in expression of PCNA and BrdU

The number of PCNA positive hepatocytes increased gradually with progression of hepatocarcinogenesis at the early stage, and showed a tendency of sharp increase after 12 wk. Two-

tailed *t* test showed that the number of PCNA positive hepatocytes of group II was lower than that of group I, and the difference between two groups was evident between 12 and 20 wk ( $P < 0.05$ ), (Table 1). The number of BrdU positively labeled hepatocytes was also smaller in group II at wk 20 of hepatocarcinogenesis ( $28.80 \pm 0.65$  vs  $45.71 \pm 1.90$ ,  $P < 0.05$ ).

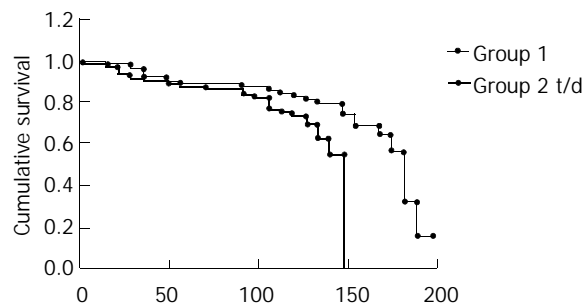
**Table 1** Effect of Ganfujian granule on positive expression of PCNA during hepatocarcinogenesis ( $n=5$ , mean $\pm$ SD)

Group	4 wk	8 wk	12 wk	16 wk	20 wk
Control	15.22 $\pm$ 3.28	21.50 $\pm$ 3.04	21.06 $\pm$ 0.32	31.10 $\pm$ 7.06	41.77 $\pm$ 1.01
Treatment	10.88 $\pm$ 3.22	12.20 $\pm$ 1.87	6.38 $\pm$ 0.18 <sup>a</sup>	10.11 $\pm$ 0.78 <sup>a</sup>	16.14 $\pm$ 0.43 <sup>a</sup>

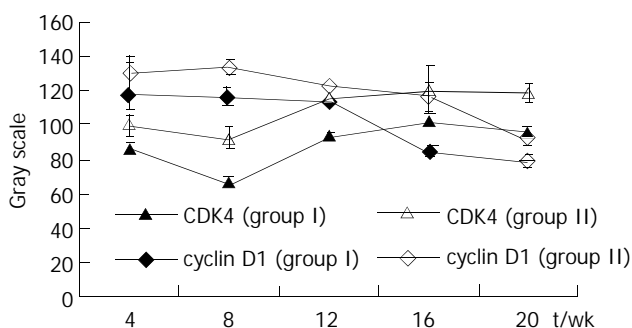
<sup>a</sup> $P < 0.05$  vs control.

### Effects of Ganfujian granule on expression tendency of cell-cycle modulators

The gray scale of positively stained cyclin D1 of hepatocytes in rats treated with DEN decreased continuously, that is, positive expression of cyclin D1 showed an increasing tendency, reaching the peak at wk 16-20 of hepatocarcinogenesis. Positive expression of CDK4 reached the peak at wk 8, decreased gradually afterwards, and rose again gradually at wk 20. Linear regression correlation analysis showed that there was no significant correlation between two modulators ( $P > 0.05$ ). Positive expression of CDK4 in group II was significantly lower than that in group I during different stages of hepatocarcinogenesis ( $P < 0.05$ ) and the expression of cyclin D1 was significantly lower at wk 12 and 20 ( $P < 0.05$ ), (Figure 2).



**Figure 1** Survival of rats in two groups.



**Figure 2** Expression of cyclin D1 and CDK4 during hepatocarcinogenesis in two groups of rat's liver.

## DISCUSSION

Carcinogenesis is a multi-stage process characterized by continuous change in specific heredity and phenotype. The whole process of carcinogenesis is accompanied by subsequent activation of a group of protooncogenes and inactivation of



cancer suppressor genes, leading to continuous accumulation of uncontrolled proliferation of cells<sup>[8]</sup>. Therefore, the liver backgrounds such as nodular cirrhosis and viral hepatitis, and abnormal expression of related genes and proteins play crucial roles in the process of carcinogenesis. In our model of using DEN as the chemical carcinogen, the rat liver underwent different stages of inflammatory change, cirrhosis, carcinogenesis and progression. The incidence rate of liver cancer was 100%. Almost all these liver cancers developed on the basis of cirrhosis. This process might imitate the pathogenesis of human liver cancer to some extent<sup>[9,10]</sup>. Therefore, our model provides necessary evidence for dynamic observation of the whole process of hepatocarcinogenetic evolution from cancer initiation to malignant transformation based on the change of cell proliferation modulations.

Ganfujian granule is composed of dietary and medicinal Chinese herbs including Chinese yam (*Rhizoma Dioscoreae*), hawthorn fruit (*Fructus Crataegi*) and Chinese date (*Fructus Ziziphi Jujubae*). It is atoxic for long-term use. As a basic formula, it has achieved good therapeutic effects in clinical treatment of liver diseases. In the present study, we observed its effect on DEN induced liver cancer in rats and found that in group I 100% of these rats presented with nodular cirrhosis at wk 16, of which 20% were accompanied by early cancer change at wk 20, all rats developed liver cancer. The longest survival of rats was 20 wk. In group II, cirrhosis was found in 60% rats at wk 16, liver cancer was found in 80% at wk 20. The longest survival of rats was 28 wk. Statistical results showed that Ganfujian granule reduced and delayed the occurrence of liver cancer in rats and prolonged survival of these animals.

Abnormal proliferation of cells is the main feature of carcinogenesis, and therefore exploration of drugs that can affect malignant proliferation of liver cells is of primary importance in chemical prevention of liver cancer. PCNA, a polypeptide chain derived and purified from the cell nucleus, is directly involved in DNA replication. It has been found that the content and positive expression of PCNA were a common index for proliferation of hepatocytes at late G1 stage and early S stage<sup>[11]</sup>. As BrdU may seep into cells during DNA synthesis, it can be used to reflect the number of S stage cells. Both PCNA and BrdU are good indexes reflecting proliferation of cells. The positive expression of two proteins was mainly found in the pre-cancerous proliferation focus and cancerous liver tissue during DEN induced hepatocarcinogenesis<sup>[9]</sup>. Their high expression suggested that the ability of cell proliferation became stronger, and this was closely related to malignant cell proliferation and carcinogenesis<sup>[12,13]</sup>. In the early stage of our experimental model, PCNA positive hepatocytes increased gradually and this increasing tendency became more evident between 12 and 20 wk (the period during which cirrhosis-liver cancer formed). The inhibitory effect of Ganfujian granule on PCNA positive cells was most prominent during this period. In addition, Ganfujian granule also reduced the number of BrdU positively labeled cells at wk 20, suggesting that Ganfujian granule had the action to suppress malignant proliferation of hepatocytes in experimental liver cancer.

The events of cell cycle are normally controlled by the clock within the cell, which is reflected by periodic activation of cyclin D/CDK complex. Irregular regulation may lead to abnormal cleavage of cells and is closely related to malignant transformation of cells<sup>[12-15]</sup>. In our experiment, we found that positive expression of cyclin D1 tended to increase gradually during DEN induced hepatocarcinogenesis, reaching the peak

at the end stage of carcinogenesis. On the other hand, positive expression of CDK4 was active during the early stage of carcinogenesis, became weaker during the middle stage, and rose gradually in the late stage. In group II, positive expression of CDK4 at various stages of carcinogenesis and that of cyclin D1 at wk 16 were significantly lower than those in group I. These findings indicate that DEN induced hepatocarcinogenesis was accompanied by continuous accumulation of cell cycle positive modulators such as cyclin D and CDK, leading to disturbance of cell cycle and uncontrolled proliferation. Ganfujian granule can suppress over-expression of these modulators, regulate the process of cell cycle and capture over-proliferation of hepatocytes that escape the G1-S check-point so as to suppress uncontrolled proliferation and hepatocarcinogenesis.

Based on the results from our experiment, it is concluded that the dietary and medicinal formula Ganfujian granule is able to reduce and delay the occurrence of liver cancer by affecting the abnormal expression of multiple proteins related to cell proliferation cycle at different stages of hepatocarcinogenesis. As a liver cancer chemical preventive agent, clinical application of Ganfujian granule is promising.

## REFERENCES

- 1 **Lemoine A**, Azoulay D, Jezequel M, Debuire B. Hepatocellular carcinoma. *Pathol Biol* 1999; **47**: 903-910
- 2 **Wang QH**, Liu XF. Study on monitoring of high risk populations of liver cancer. *Guowai Yixue Zhongliu Fence* 2002; **29**: 449-451
- 3 **Chang MH**, Chen DS. Prospects for hepatitis B virus eradication and control of hepatocellular carcinoma. *Baillieres Best Pract Res Clin Gastroenterol* 1999; **13**: 511-517
- 4 **Baffis V**, Shrier I, Sherker AH, Szilagyi A. Use of interferon for prevention of hepatocellular carcinoma in cirrhotic patients with hepatitis B or hepatitis C virus infection. *Ann Intern Med* 1999; **131**: 696-701
- 5 **Young KJ**, Lee PN. Intervention studies on cancer. *Eur J Cancer Prev* 1999; **8**: 91-103
- 6 **Li ZQ**. Traditional Chinese medicine for primary liver cancer. *World J Gastroenterol* 1998; **4**: 360-364
- 7 **Qian Y**, Ling CQ, Yu CQ, Pan RP, Zhang YN, Wang YZ. Obstructive effect of three traditional Chinese prescriptions on liver preneoplastic lesion in rats. *Disi Junyi Daxue Xuebao* 1999; **20**: 916-918
- 8 **Wei W**, Gong JP, Qiu FZ. Relationship between cell proliferation and apoptosis during the normal and abnormal liver tissue proliferation. *Zhonghua Shiyian Waikexue Zazhi* 2001; **18**: 156-158
- 9 **Zhang XL**, Shi JQ, Bian XW. Quantitative study on morphologic features and proliferative activity during DEN induced hepatocarcinogenesis in rats. *Disi Junyi Daxue Xuebao* 2001; **23**: 304-307
- 10 **Ling CQ**, Qian Y, Zhao JA, Jin Y. Expression of c-myc IGF-II gene and CycinD1 protein in experimental hepatocarcinoma. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 1452-1453
- 11 **Lu S**, Zhan AH, Huang XX, Ren YJ. Expression and significance of proliferating cell nuclear antigen in resistance of lithium carbonate to Aflatoxin B1 induced hepatocarcinogenesis in rats. *Aibian Jibian Tubian* 2002; **14**: 84-86
- 12 **Kui Y**, Lin C, Wu W. Relationship between several important modulators of cell cycle G1-S check point and tumor. *Guowai Yixue Fenzi Shengwuxue Fence* 1998; **20**: 10-13
- 13 **Liu LX**, Jiang HC, Zhu AL, Wang XQ, Zhu J. Cell cycle and growth regulators gene expression in liver cancer tissues and adjacent normal tissues. *Zhonghua Shiyian Waikexue Zazhi* 2001; **18**: 123-126
- 14 **Yuan JH**, Zhong RP, Zhong RG, Guo LX, Wang XW, Luo D, Xie Y, Xie H. Growth-inhibiting effects of taxol on human liver cancer *in vitro* and in nude mice. *World J Gastroenterol* 2000; **6**: 210-215
- 15 **Yang LJ**, Si XH. Expression and significance of cyclin D1 in human hepatocellular carcinoma. *Shiyong Zhonggluxue Zazhi* 2000; **15**: 124-126

# Definitive palliation for neoplastic colonic obstruction using enteral stents: Personal case-series with literature review

Giuseppe Piccinni, Anna Angrisano, Mario Testini, G. Martino Bonomo

**Giuseppe Piccinni, Anna Angrisano, Mario Testini, G. Martino Bonomo**, Section of General Surgery, Vascular Surgery and Clinical Oncology, Department of Applications in Surgery of Innovative Technologies, University of Bari- School of Medicine - Bari, Italy  
**Correspondence to:** Giuseppe Piccinni M.D., Sezione di Chirurgia Generale, Vascolare ed Oncologia Clinica, Dipartimento per le Applicazioni in Chirurgia delle Tecnologie Innovative Università di Bari, Policlinico, Piazza G. Cesare 11, 70124, Bari, Italy. [bepppiccinni@tin.it](mailto:bepppiccinni@tin.it)  
**Telephone:** +39-080-5478856 **Fax:** +39-080-5478749  
**Received:** 2003-10-27 **Accepted:** 2003-12-24

## Abstract

Acute colonic obstruction due to malignancies is an emergency that requires surgical treatment. Elderly patients or inoperable tumors require intestinal decompression that is a simple colostomy in almost all cases. This "manoeuvre" leads the patient to a percentage of mortality/morbidity and to a bad quality of life due to acceptance of stoma. The introduction of enteral metal stent inserted endoscopically has, in our opinion, provided a new way to obtaining the definitive palliation of inoperable colo-rectal cancer with a simple method. We reported our case-series and we analyzed the current literature and costs of treatments.

Piccinni G, Angrisano A, Testini M, Bonomo GM. Definitive palliation for neoplastic colonic obstruction using enteral stents: Personal case-series with literature review. *World J Gastroenterol* 2004; 10(5): 758-764

<http://www.wjgnet.com/1007-9327/10/758.asp>

## INTRODUCTION

Curative resection of colorectal cancer is not feasible in more than 25% of patients presenting obstruction due to extensive local tumor infiltration, distant metastases, or severe comorbidities.

In the treatment of patients with inoperable malignant obstruction, maintenance of gastrointestinal luminal patency is of paramount importance, and in view of this palliative cures are challenging.

Surgical treatment still remains the optimal treatment for high-risk patients with large bowel obstruction, although morbidity and mortality rates are relatively high. Moreover, surgical decompression with a palliative colostomy may be a major source of morbidity, and increase the time of hospitalization, need for further medical care, and reduces quality of life. In the last decade the health care industry has introduced really sophisticated visceral prosthesis. In recent years we have been challenged in stenting occlusive colo-rectal cancer bridging patients from emergency to elective surgery. With this work we reported our experience in treating inoperable colonic neoplastic obstruction, proposing definitive palliation for both obstructive symptoms and continuous oral intake, positioning enteral stents. This endoscopic treatment, in high-risk or inoperable patients, can avoid surgical approach with colostomy, greatly enhancing the quality of life.

Evaluation of the outcome of this treatment cannot ignore cost analysis that appears favorable in our and other opened experiences.

## CASE SERIES

A retrospective review performed on 4 patients presenting incomplete colonic obstruction secondary to left-side colon cancer received a colonic stent at our institution between June 2000 and July 2003.

Clinical and radiographic criteria for patient eligibility to this treatment included (a) symptoms of obstruction with constipation for a period longer than 48 h, abdominal distension, nausea, vomiting, or abdominal pain, and (b) conventional radiologic evidence of colon-rectal obstruction (confirmed by abdominal computed tomography).

We excluded patients if they manifested clinical evidence of bowel perforation and free intraperitoneal air on abdominal radiograph, peritonitis, massive gastrointestinal bleeding, a fixed rectal mass.

All patients underwent baseline endoscopic evaluation for delineation of tumor length and demarcation. In all cases, histopathologic findings from biopsy revealed adenocarcinoma. After adequate explanation regarding discouraging complications (including difficulty of insertion because of propagation of the tumor, possible perforation on insertion and expansion of the stent, stent migration and tumor ingrowth), informed consent was given by each patient and family.

## Case 1

In July 2000 a 90-year-old man came to our department because of rectal bleeding, appearing 1 mo before, and ileus symptoms. At admission the physical examination demonstrated abdomen distension and a rectal mass on finger exploration. Laboratory tests revealed an anaemic condition alone. American Society of Anesthesiologists (ASA) status was III. Then the patient underwent nasogastric decompression and received intravenous fluid supplements. CT of the abdomen and pelvis showed a rectal mass involving the whole wall thickness. Colonoscopy identified a substenotic rectal mass, measuring 4 cm in length, 8 cm away from the dentate line, which was dilated with a TTS-balloon (BE-6 OLYMPUS - Europe, Amburg, Germany). We decided to stent the lesion and opted for this kind of temporary treatment because of stent supply times. Balloon dilation showed an improvement of abdominal symptoms with gas and liquid stools transit. In the meantime we supported the patient with a liquid diet and total parenteral nutrition. Small enemas were also administered. On d 10 after admission, we inserted a Wallstent prosthesis (Schneider, Bulach, Switzerland) measuring 9 cm in length and 22 mm in diameter. The patient's symptoms improved immediately after stent placement with passage of stools and flatus from the anus. He died 6 mo after stent placement due to progression of the initial disease without constipation symptoms or signs.

## Case 2

In July 2002 a 75-year-old man was admitted complaining of

abdominal pain and constipation for 3 d. Family history was positive for familiarity with neoplastic colonic disease (one sister had died). Past history: in 1984 radical cystectomy with urostomy for bladder carcinoma, chronic renal failure, in 1994 surgical clearing for choledocholithiasis, cerebral ischaemia and outcomes of lacunar encephalopathy, left hemiparesis, sclero-hypertensive cardiomyopathy, ventricular extrasystoles. In the last 6 mo, a body mass loss of 10 kg and poor appetite were noted. The patient had also been suffering from hypogastric pain for a month. For this reason, on 27 of July 2002 he underwent colonoscopy that revealed a circular stenosis 75 cm away from the anal verge, in the distal transverse colon (near splenic flexure) line, and unable to get through. Biopsy of this tissue demonstrated a histopathological finding of a well-differentiated adenocarcinoma. On August 28, 2002 he was referred to our institution because of ileus symptoms.

At physical examination, his abdomen was markedly distended. Laboratory tests disclosed hyper blood urea and hyper-creatinine level, malnutrition with a total protein level of 5.4 g/dL (normal value 6.4-8.3 g/dL), a white blood cell count of 8.500/mm<sup>3</sup> (with normal differential cell and platelet counts), a hemoglobin level of 12 g/dL, a hematocrit of 36%. The ASA status was IV.

Plain abdominal Rx at admission showed fluid line. He was then subjected to total parenteral nutrition and a nasogastric tube to reduce gastrointestinal pressure. On the following days, ileus was temporally improved, on September 4, he underwent definitive colonic decompression using a Wallstent (Boston Scientific Microvasive, Minnesota, USA) measuring 9 cm in length and 22 mm in diameter (Figure 1). After one year he is still alive, follow-up control was scheduled for the 30<sup>th</sup> 2003.

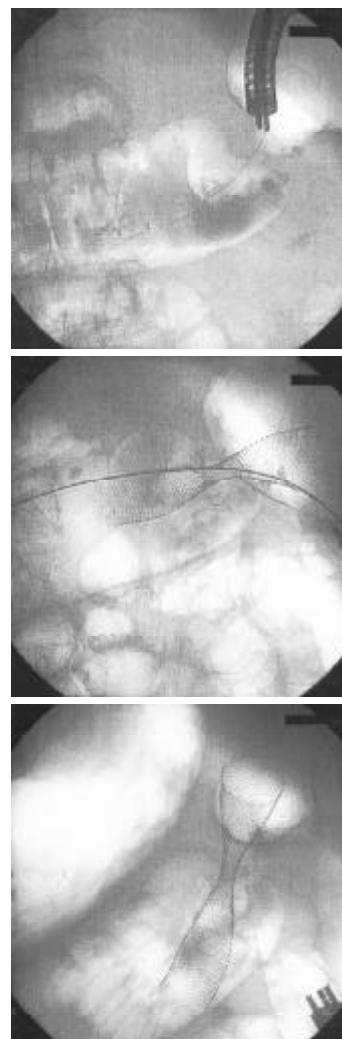
### Case 3

An 85-year-old woman was admitted to our department in October 2002 suffering from rectal bleeding first noted the year before. On admission the patient was diagnosed with ileus symptoms due to ulcerated, exophytic substenotic recto-sigmoid neoplasm, 5 cm long starting 14 cm from the anal verge as a result of colonoscopic examination. Laboratory studies disclosed malnutrition (total protein level of 5.6 g/dL) and anaemia (hemoglobin level of 10.7 g/dL, hematocrit of 33.5%). ASA status was IV. Plain computed tomography revealed tumor shadow of the sigmoid and the cephalic side of the colon was markedly dilated with fluid collection. Swelling of intraperitoneal lymph nodes and an abundant bilateral pleural effusion were also noted. We inserted a Precision Ultraflex prosthesis (Boston Scientific Microvasive, Minnesota, USA), measuring 9 cm in length and 25 mm in diameter. We obtained immediate recovery of colonic transit (Figure 2). She was discharged after 2 d and died after 45 d.

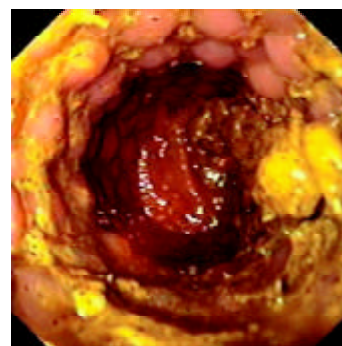
### Case 4

A 61-year-old woman, without any medical history, had its beginning in March 2003, when she noted relapsing episodes/events of sub-occlusion and a body mass loss of 10 kg. At hematologic examinations, carcino-embryonic antigen and carbohydrate antigen 19-9 levels were significantly elevated at 8 000 ng/mL and over 10 000 U/mL, respectively. Plain computed tomography performed on May 29 showed multiple liver metastases, ascites and peritoneal carcinomatosis. Colonoscopic examination showed a circular stenosis at the sigmoid. Histopathological findings demonstrated adenocarcinoma. Initial support therapy was sought at a medical institute and then she was referred to ours for the required treatment. ASA-status was IV. A water-soluble contrast-media enema performed just after the colonoscope introduction, demonstrated a 3 cm

long stenosis. A Precision Ultraflex stent, measuring 9 cm in length and 25 mm in diameter, was selected and positioned. The day after stent insertion, we saw a full canalization. The patient was discharged on the 3rd hospital day and she is still alive (end of follow-up: August 2003).



**Figure 1** Radiologic sequence of stenting procedure in left transverse colon.



**Figure 2** Endoscopic view of Wallstent positioned in the sigmoid colon with recovery of intestinal transit.

Before stent placement, all the patients underwent one or more colonic enemas, depending on the site of obstruction for cleaning the colon below the stricture. The patients were placed in the supine position only using a suitable combination of intravenous medication appropriate for a colonoscopic procedure as sedation and analgesia. There was no routine

administration of antibiotics. Vital signs (pulse rate and oxygen saturation) were monitored continuously.

As regards the stent placement technique used, once the colonoscope was inserted, a water-soluble contrast media was injected for careful assessment of the length and the morphology of the stricture by fluoroscopy. Then, an Amplatz superstiff guidewire (Boston Scientific Microvasive, Minnesota, USA) was inserted through the colonoscope channel and advanced as far as possible into the proximal bowel, and kept *in situ*. The instrument was then removed and reinserted adjacent to the wire. The stent was then deployed over the guidewire and across the stenosis. Using fluoroscopy, the stent was placed with each end equidistant from the tumor margins except for the first case. Deployment was performed under fluoroscopic and endoscopic guidance. Finally, endoscopic and X-ray images were used to assess the accuracy of the stent position.

Dedicated enteral stents were used (Enteral Wallstent/Precision Ultraflex endoprotheses). We chose enteral stents about 5 cm longer than the stricture, 1 or 2 cm of the stent to extend beyond both ends of the tumor.

We did not dilate the strictures before stent placement, except for the first patient, because neoplasms were not totally obstructing, but it should be remembered that this procedure could increase the risk of colonic perforation.

To determine the stent position and the relief of colonic obstruction until the patients were discharged, a conventional radiograph of the abdomen was obtained and changes in bowel gas patterns were analyzed within 24 h of stent placement and each subsequent day thereafter.

All patients tolerated the procedures well, which were technically successful.

There were no immediate technical complications associated with stent placement, and no perforation, major bleeding, or death related to endoscopic procedure. Each of the patient's symptoms improved immediately after stent placement with passage of stools and flatus from the anus. Initially the patients were able to take at least liquids, later they assumed a low-residue diet. The only delayed complication was dislodgment of 1 stent (25%) after one month.

The average survival time was 22.5 wk, and ranged from 6 to 48 wk. Two patients were alive at the end of the study period, two died because of the natural disease history. No patients showed clinical symptoms of obstruction at the time of death or termination of the study, all tolerated oral semi-solid feedings.

## DISCUSSION

Malignancy is the major cause (85%) of acute colonic obstruction (ACO) and 10-30% of patients with colonic cancer had a large-bowel intestinal occlusion at presentation<sup>[1-3]</sup>.

Therefore, not surprisingly, ACO is considered as a surgical emergency, traditionally treated with surgical intervention that, though effective, was associated with high morbidity (10-36%) and mortality (6-30%) rates<sup>[4]</sup>.

Emergency surgery, mainly in acutely ill patients, often results in a diverting colostomy alone, which may be associated with a morbidity rate of 20-40% and a mortality rate of more than 10%<sup>[5]</sup>.

Indeed, many of these patients have already reached an advanced stage of the disease at the time of diagnosis. For patients with advanced tumors no longer resectable, widespread metastatic disease, peritoneal carcinomatosis, unfortunately the only therapeutic options have been palliative, with colostomy being the only reasonable and often unavoidable surgical option<sup>[6]</sup>. Besides, many of these patients were elderly (up to 50% between 70 and 89 years old) and instable because of

significant co-morbidities, all this made them of high surgical risk<sup>[7-9]</sup>. Moreover this treatment option entailed a significant decrease in quality of life, with major psychological repercussions<sup>[10]</sup>. In addition, this unfortunate group of patients had short life expectancies (mean survival of patients who had hepatic metastases at the time of surgery was only 4.5 mo)<sup>[11]</sup> and it was of paramount importance to keep them out of hospital. So, it is therefore preferable to seek more comfortable therapeutic approaches.

The primary goal of a non-surgical approach for treating ACO is to avoid the need of emergency surgical treatment in non stabilized patients. Such non-surgical alternatives to colostomy as balloon dilation<sup>[12]</sup> and ablative methods (cryotherapy, electrocoagulation, laser photocoagulation)<sup>[13,14]</sup>, have been used in inoperable patients or with unresectable tumors. Laser therapy (LT) has gained considerable support by virtue of reports of its high initial success in luminal diameter increasing. Nd:Yag laser therapy was considered the treatment of choice for endoscopic palliation of advanced rectal carcinoma. In 1986 Mathus-Vliegen reported a success rate of 85-95% (5, 15). The drawbacks of LT were expensive equipment, time consuming sessions, lack of immediate relief of symptoms, need for repeated sessions to maintain patency, needs to repeat sessions, even every 5 to 9 wk<sup>[12,16,17]</sup>, significant (13%)<sup>[18,19]</sup> risk of major complications like stenosis, perforation<sup>[20]</sup>, fistula (3.2%), abscess (1.7%) and bleeding (4.1%), as published by Gevers *et al*<sup>[21]</sup>.

In conclusion, LT is affected by such shortcomings as the need to be repeated periodically to maintain patency and limited applicability restricted solely to selected patients<sup>[22]</sup> with tumors in distal locations.

In recent years, enteral stenting has emerged as an effective alternative to the surgical approach. However, the concept of colonic decompression using "stent" in obstructing colonic tumors, in the absence of peritonitis<sup>[23]</sup>, is not new. Initial reports were published in the early 1990s with some promising results but containing only a handful of patients, Lelcuk in 1986<sup>[24]</sup> and Keen<sup>[25]</sup> in 1992 passed a nasogastric tube respectively through the tumor to relieve obstruction. In 1991 the first report describing use of a metal stent in the rectum was published by Dohmoto<sup>[26]</sup>. The following year, in a small series of four and two patients respectively, Spinelli in 1992<sup>[27]</sup> and Itabashi in 1993<sup>[28]</sup> confirmed the feasibility of inserting self-expanding metal stents for immediate relief of acute colonic obstruction due to rectal malignant tumors. In 1998 De Gregorio<sup>[29]</sup> published a large multicenter retrospective study (24 pts) evaluating the success of the placement of colorectal stents for palliation, reporting a 100% technical success and a 96% clinical improvement. Using various kinds of stent, in 25 patients, Baron's group<sup>[30]</sup> had less impressive results. They failed to put in the stents in 6% of their patients because of technical problems and only 85% of their stented patients had relief from obstruction.

The question as to which strictures by site are amenable to colonic endolumenal stenting (CELS) is not well addressed in literature by many authors. Similarly their description of the sites of lesions they have stented is unclear. The most commonly reported cases of CELS in literature were of lesions within the rectum and rectosigmoid, which was unsurprising as 70% of the obstructing colonic strictures were located in the left colon<sup>[1,31]</sup>.

While the initial series excluded patients thought to have lesions located in difficult anatomical positions<sup>[32]</sup>. To date location has not appeared to be a limitation for CELS<sup>[30,33,34]</sup>, neither did the length of the tumor. If the lesion was not successfully or totally covered by the stent, an additional stent could be placed.

Distal extent of disease does not limit suitability for

stenting, although lesions less than 5 cm from the anal verge (dentate line) might be inappropriate<sup>[35,36]</sup> and difficult to palliate with a metal stent, because of perianal trauma occurring due to stent irritation<sup>[37]</sup>. Despite the recent advances in stent technology, the search for the ideal enteral stent has not stopped. Enteral stents should be flexible enough to allow placement but should remain in position once deployed<sup>[38]</sup>.

Initially since dedicated colonic stents were not available, a variety of stents originally designed for use elsewhere were used, as reported in literature studies, including the preferred enteral Wallstent®<sup>[39-41]</sup>. Others were Endocoil® (Euromed Inc), esophageal nitinol Strecker stent, Gianturco self-expanding Z-stent, Ultraflex® Instent Esophacoil®<sup>[28,30,34,39,40,42-45]</sup>. There was no noticeable difference in the outcomes using any of the above stents.

Metallic stents are of two types, expandable or self-expanding (so-called self-expanding metal stents, or SEMS). Because of their flexibility SEMS are easier to deploy than rigid tubes and allow peristalsis to continue, but they usually have a narrow lumen and are prone to tumor ingrowth. Some of these mesh type stents have been coated with polyurethane or other materials to prevent occlusion by tumor although this may increase the likelihood of dislocation. Covered stents have the advantage of resisting tumor ingrowth but tend to be less stable and more rigid, they require a larger delivery system, and are more likely to migrate. They are thus more difficult to deploy at distant locations through a tortuous delivery path<sup>[46]</sup>. Uncovered stents are more flexible, and at least one can be passed through the working channel of an endoscope. However, when used for long-term palliation of malignant obstruction, they are subjected to tumor ingrowth and resultant obstruction. Most authors now use SEMS made of nitinol, a nickel/titanium alloy, which has <<shape memory>> meaning that once deployed, it adopts a preformed shape, an advantage over expandable systems that require dilation. Stent technology is currently evolving rapidly, and devices are now being developed specifically for colorectal applications.

The mean technical success, defined as successful stent placement and deployment, ranged from 64% (40) and 100%<sup>[32,28,39,47]</sup>. Failure to stent deployment was usually a result of inability to pass a guide wire through a lesion, other reasons for technical failure, apart from tight or tortuous stenosis through which the guide wire could not be passed<sup>[30,34,40,44,45]</sup> included insufficient length of the stent to span the entire stricture, inadequate introducer lengths<sup>[30,44]</sup>, floppy introducer system and incorrect deployment of the stent (42). A higher rate of successful stent placement could be achieved with more distal lesions. The most proximally placed stents described in literature till now have been in the right and proximal transverse colon<sup>[30,34]</sup>.

The stent placement procedure is generally painless and neither anesthesia nor analgesia is provided. It was frequently sufficient to provide a suitable combination of intravenous medication for a simple colonoscopy procedure<sup>[9,17]</sup>. Clinical success, that is relief of obstructive symptoms, defined as colonic decompression within 96 h without endoscopic or surgical reintervention after stent placement (this time interval was chosen because it was integral to the definition used in nearly all the papers reviewed) has been reported in 75-96%<sup>[2,23,30]</sup>.

Stent placement complications were common to many authors, distinguishing the early from late ones. In previously published series<sup>[9,29,34,45,48]</sup> the complication rate has been reported to range from 14% to 42%, most complication being minor. In the literature surveyed these were successfully managed with medical or supportive treatment in the majority of cases. Less seen complications included minor rectal bleeding, anorectal pain, temporary incontinence (11%)<sup>[42]</sup>, fecal impaction (8%)<sup>[40]</sup> and severe tenesmus. Rectal bleeding

(0-100%) was usually hemodynamically insignificant and self-limited. It could result from mucosal irritation, pressure necrosis of the stent in the colonic mucosa, or friability of the tumor itself<sup>[49]</sup>. Anorectal pain (5-100%) might occur and was usually mild and transient, lasting for only 3-5 days, it was easily controlled by analgesia<sup>[39,45,49]</sup>. Severe tenesmus occurred during the first 48 hours and might be controlled with non-steroid anti-inflammatory drugs. It seemed to be related to insertion of the stent in a lower portion of the rectum<sup>[50]</sup>. However, Camuñez<sup>[51]</sup> described a patient who was readmitted twice because of persistent analgesic therapy, and was offered a colostomy which ultimately was refused.

Stent migration, restenosis, and perforation were the major complications encountered with colonic stent placement. Stent dislocation (0-44%) and obstruction (0-33%) were reported as the most common major complications described, but are not usually serious. Stent migration has been reported to occur in as many as 40% of cases and was usually detected on follow-up radiographs within 1 week of insertion. Camuñez<sup>[22]</sup> believed the cause of stent migration was shrinkage of the tumours as a result of adjuvant chemotherapy. Generally, it appeared that predisposing factors included inappropriate stent selection as covered stent or those with too narrow diameter (with weak radial expansive strength), colonic angulation and post-operative chemotherapy or radiation therapy<sup>[38,49,52]</sup>. Consequently, specific monitoring to check for possible reduction in tumor size and assess the advisability of stent extraction, is recommendable in patients receiving adjuvant therapy. When stent migration occurred, the stent might generally be passed spontaneously or require endoscopic removal and redeployment<sup>[49]</sup>. Some stents which became dislocated and were expelled did not necessarily require replacement, since bowel function was adequately maintained.

Restenosis has been reported in up to 25% of cases and was usually due to malpositioning of the stent, as well as impaction with stool or food matter (it has been recommended that patients with colorectal stent ingest a low-residue diet and use stool softeners to lessen the likelihood of stent obstruction)<sup>[38,53]</sup> but especially tumor ingrowth. Stent obstruction was usually amenable to a variety of nonoperative measures such as further coaxial restenting<sup>[30]</sup> and laser therapy<sup>[42]</sup>. However it would be interesting to point out that in Dohmoto's study 15% of patients needed a palliative colostomy to treat stent occlusion related to tumor ingrowth.

Restenosis due to tumor overgrowth (extension of tumor above or below the stent) could also be treated with a second stent. It could be best prevented by deploying the original stent 2-3 cm above or below the lesion when possible.

The most serious and potentially devastating complication of colonic stent placement was colonic perforation, reported in 0-16% of cases<sup>[49,54,55]</sup>. Perforation can be either early or late, it should be suspected in patients who complain of abdominal pain during or immediately after the stenting procedure. Colonic perforation may have different causes, related either to the method itself or to the stent. One of the major causes is excessive balloon dilation of the stricture, causing full-thickness tear of the mucosa. Baron *et al*<sup>[30]</sup> reported colonic perforation in four patients, associated with balloon dilation of the stricture at the time of stent placement in three of the patients. Without balloon dilation, perforation rate might fall below 5%. Therefore, this practice is no longer recommended<sup>[56]</sup>, and stents are allowed to slowly self-expand.

Fortunately, it is possible to achieve stent placement even in patients with tight strictures without employing dilatation when modern stents are used. In addition, as perforation is more likely to occur during guidewire manipulation, the introduction of a wide range of soft-tipped guide-wires may contribute further to prevention of perforation. In the experience

of many authors<sup>[34,44,22]</sup> perforation due to manipulation of guidewires and catheters has been asymptomatic, and it has been possible to complete the procedure in all cases. Late perforation could occur (though it is rare) due to pressure necrosis and erosion through the colon. Histologically, the side of the cancerous lesion compressed by the stent was thin and consisted of a serosal layer alone. Granulomatous change was detected histologically in normal mucosa that was in contact with the metallic stent. These histological changes were caused by direct compression or ischemic damage due to the self-expanding stent. Therefore, if SEMS is inserted in patients with complete obstruction due to advanced carcinoma infiltrating the serosa, Kusayanagi's experience suggests that a risk of perforation might develop approximately 2 months later<sup>[49,57]</sup>. Several authors concluded that the possibility of colonic perforation as a potential complication must be kept constantly in mind and that consequently close clinical observation was required particularly in the days immediately following stent placement<sup>[22]</sup>.

Because of the continual changes in design, it is difficult to compare complication rates among different kinds of stent. However, some general conclusion may be drawn. The decreasing diameters of delivery systems make perforation a rare occurrence, and it is generally related to pre-stenting treatments.

Indeed, certain authors, prior to stent placement, pretreated the neoplastic strictures endoscopically first to canalize an obstruction using laser therapy<sup>[17,48]</sup>, argon plasma or coagulation, others used mechanical dilatation with endoluminal balloon catheters<sup>[30,41]</sup>. Nevertheless, the added efficacy of these approaches is unproven, and certainly they increase the risk.

Patients stented for palliation usually underwent clinical follow-up, conventional abdominal radiographs were obtained at monthly intervals. Period of patency was defined as the period from stent placement to the recurrence of symptoms of obstruction in clinically successful cases<sup>[58]</sup>. No absolute data exist on the mean time to occlusion of stents, also because this procedure has been carried out in a small total number of patients in the same short follow-up periods. Hence, assessment of stent patency and long-term behavior are difficult to define and data coming from literature are wide-ranging. Thus, according to Baron<sup>[30]</sup>, stent duration ranged from 2 to 64 wk (mean 17.3 wk). Kusayanagi's data<sup>[57]</sup> was also in this range. According to him the mean time before re-obstruction was approximately 10 wk and the median follow-up for Repici<sup>[59]</sup> was 21 wk (range 1-46). Moreover, no recurrence of obstruction was observed during the follow-up period. For Camuñez<sup>[51]</sup> follow-up lasted an average of 138±93 d (range: 36-334 d), and the estimated primary stent patency rate was 91% at 3 and 6 mo. Even for mortality rate existing data in literature are controversial. For some authors<sup>[17,60]</sup> no deaths have been reported directly attributable to the stenting procedure, on the contrary, Spinelli<sup>[48]</sup> had a mortality rate of 3%.

Until now we have seen that, when LBO due to cancer has progressed beyond the stage of any curable intervention, there are only two options to relieve the obstruction by colostomy or by use of a stent, since both are capable of relieving acute obstruction and stenting clearly offers a preferable quality of life. Another important issue to consider is their cost, considering the fact that the principal cost determinants for the stenting option was the cost of the stent itself and the duration of in-hospital treatment<sup>[61,62]</sup>. Few publications examined the cost impact of stenting compared to surgical decompression. One study<sup>[34]</sup>, concerning the cost of the stenting procedure compared with a control group undergoing only surgical treatment for malignant colorectal obstruction, showed that the overall cost of treating the stented group was 19.7% lower. According to Zollikofer's data<sup>[63]</sup>, a British

study<sup>[64]</sup>, comparing the cost of management by stenting 16 patients with acute large bowel obstruction with 10 unselected patients previously managed by surgical decompression, estimated that the cost of a palliative care was less than half of that of a surgically decompressed case. These cost savings were due mainly to the shorter hospital stay associated with stenting. Other factors were fewer surgical procedures, reduced operating time and fewer days in intensive care room. On the other hand, these data have much more relevance considering that the often hidden cost of stoma care in the community cannot be overlooked, about Euro 100 per mo is required for disposable stoma bags for each patient.

## CONCLUSION

Malignant neoplasm of the left colon and rectum may lead to bowel obstruction in 10% to 30% of cases. Acute malignant obstruction of the large bowel is considered as a surgical emergency. Controversy currently exists as to optimal approach among surgical and nonoperative techniques in the case of patients with unresectable cancer or with a high ASA-grade. Physicians are often faced with the dilemma of finding the right balance between the need for timely aggressive intervention and the patient's underlying debilitated state. Recently several reports have indicated that, to reestablish luminal patency, the use of a stent may be a valid alternative to traditional surgical approach often consisting of a definitive colostomy. This treatment option entails serious disadvantages, namely substantial morbidity, and an obvious decrease in quality of the remaining life, with important psychological repercussions. The stent insertion is a combined endoscopic-radiologic technique that enables decompression of acute colonic obstruction with immediate restoration of bowel function, allowing all the pathophysiologic changes of obstruction to reverse. This procedure is feasible and successful in fully relieving the obstruction in almost all cases. Neither balloon dilation nor laser therapy is universally recommended prior to colorectal stenting to canalize a neoplastic obstruction, because of unproven efficacy and certainly increased risk.

We believe that the advantages of minimally invasive management of malignant acute large bowel obstruction are of particular and clear interest for geriatric population, because many of them often are not particularly good surgical candidates and so they are not suitable for definitive curative surgery.

In our experience there is evidence that is possible to stent a tumor in more proximal difficult tract like the left transverse colon near the splenic flexure. In the future stent technology and design will continue to evolve with the aim of improving safety parameters. In addition to this, the suggestion that stents impregnated with chemotherapeutic agents or composed by radioactive wire, may also help the tumor growth control, represents a very exciting area of interest.

Surgeons should become familiar with this technique, since it offers an alternative to surgical therapy for patients in critical general conditions, improving their quality of life and reducing the costs of the whole treatment. Therefore, we recommend that enteral stenting be considered as an alternative to traditional surgical therapy for definitive palliation in patients with malignant enteral obstruction.

## ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of Professor Malcolm Clark in the preparation of the English manuscript.

## REFERENCES

- 1 Deans GT, Krukowski ZH, Irwin ST. Malignant obstruction of



- the left colon. *Br J Surg* 1994; **81**: 1270-1276
- 2 **Khot UP**, Lang AW, Murali K, Parker MC. Systematic review of the efficacy and safety of colorectal stents. *Br J Surg* 2002; **89**: 1096-1102
- 3 **Nicholl MB**, Shilyansky J, Ota DM. Current management of malignant large-bowel obstruction. *Contempor Surg* 2002; **58**: 396-400
- 4 **Riedl S**, Wiebelt H, Bergmann U, Hermanek P Jr. Postoperative complications and fatalities in surgical thera of colon carcinoma. Results of the german multicenter stud by the colorectal carcinoma study group. *Chirurg* 1995; **66**: 597-606
- 5 **Dohmoto M**, Hunerbein M, Schlag PM. Palliative endoscopic therapy of rectal carcinoma. *Eur J Cancer* 1996; **32A**: 25-29
- 6 **Phillips RK**, Hittinger R, Fry JS, Fielding LP. Malignant large bowel obstruction. *Br J Surg* 1985; **72**: 296-302
- 7 **Anderson JH**, Hole D, McArdle CS. Elective versus emergency surgery for patients with colorectal cancer. *Br J Surg* 1992; **79**: 706-709
- 8 **Scott-Conner CE**, Scher KS. Implications of emergency operations on the colon. *Am J Surg* 1987; **153**: 535-540
- 9 **Mainar A**, De Gregorio MA, Ariza MA, Tejero E, Tobio R, Alfonso E, Pinto I, Herrera M, Fernández JA. Acute colorectal obstruction: treatment with self-expandable metallic stents before scheduled surgery—results of a multicenter study. *Radiology* 1999; **210**: 65-69
- 10 **Nugent KP**, Daniels P, Stewart B, Patankar R, Johnson CD. Quality of life in stoma patients. *Dis Colon Rectum* 1999; **42**: 1569-1574
- 11 **Bengtsson G**, Carlsson G, Hafstrom L, Jonsson PE. Natural history of patients with untreated liver metastases from colorectal cancer. *Am J Surg* 1981; **141**: 586-589
- 12 **Oz MC**, Forde KA. Endoscopic alternatives in the management of colonic strictures. *Surgery* 1990; **108**: 513-519
- 13 **Hoekstra HJ**, Verschueren RC, Oldhoff J, van der Ploeg E. Palliative and curative electrocoagulation for rectal cancer. Experience and results. *Cancer* 1985; **55**: 210-213
- 14 **Spinelli P**, Dal Fante M, Meroni E. Traitement endoscopique au laser des tumeurs colo-rectales. *Acta Endosc* 1987; **17**: 157-168
- 15 **Mathus-Vliegen EM**, Tytgat GN. Laser photocoagulation in the palliation of colorectal malignancies. *Cancer* 1986; **57**: 2212-2216
- 16 **Dauphine CE**, Tan P, Beart RW Jr, Vukasin P, Cohen H, Corman ML. Placement of self-expanding metal stents for acute malignant large-bowel obstruction: a collective review. *Ann Surg Oncol* 2002; **9**: 574-579
- 17 **Harris GJ**, Senagore AJ, Lavery IC, Fazio VW. The management of neoplastic colorectal obstruction with colonic endoluminal stenting devices. *Am J Surg* 2001; **181**: 499-506
- 18 **Russin DJ**, Kaplan SR, Goldberg RI, Barkin JS. Neodymium-YAG laser. A new palliative tool in the treatment of colorectal cancer. *Arch Surg* 1986; **121**: 1399-1403
- 19 **Wodnicki H**, Goldberg R, Kaplan S, Yahr WZ, Kreiger B, Russin D. The laser: an alternative for palliative treatment of obstructing intraluminal lesions. *Am Surg* 1988; **54**: 227-230
- 20 **Mathus-Vliegen EM**, Tytgat GN. Analysis of failures and complications of neodymium: YAG laser photocoagulation in gastrointestinal tract tumors. A retrospective survey of 18 years' experience. *Endoscopy* 1990; **22**: 17-23
- 21 **Gevers AM**, Macken E, Hiele M, Rutgeerts P. Endoscopic laser therapy for palliation of patients with distal colorectal carcinoma: analysis of factors influencing long-term outcome. *Gastrointest Endosc* 2000; **51**: 580-585
- 22 **Camunez F**, Echenagusia A, Simo G, Turegano F, Vazquez J, Barreiro-Meiro I. Malignant colorectal obstruction treated by means of self-expanding metallic stents: effectiveness before surgery and in palliation. *Radiology* 2000; **216**: 492-497
- 23 **Tamin WZ**, Ghellai A, Counihan TC, Swanson RS, Colby JM, Sweeney WB. Experience with endoluminal colonic wall stents for the management of large bowel obstruction for benign and malignant disease. *Arch Surg* 2000; **135**: 434-438
- 24 **Lelcuk S**, Ratan J, Klausner JM, Skornick Y, Merhav A, Rozin RR. Endoscopic decompression of acute colonic obstruction. Avoiding staged surgery. *Ann Surg* 1986; **203**: 292-294
- 25 **Keen RR**, Orsay CP. Rectosigmoid stent for obstructing colonic neoplasms. *Dis Colon Rectum* 1992; **35**: 912-913
- 26 **Dohmoto M**, Rupp KD, Hohlbach G. Endoscopically-implemented prosthesis in rectal carcinoma. *Dtsch Med Wochenschr* 1990; **115**: 915
- 27 **Spinelli P**, Dal Fante M, Mancini A. Self-expanding mesh stent for endoscopic palliation of rectal obstructing tumors: a preliminary report. *Surg Endosc* 1992; **6**: 72-74
- 28 **Itabashi M**, Hamano K, Kameoka S, Asahina K. Self-expanding stainless steel stent application in rectosigmoid stricture. *Dis Colon Rectum* 1993; **36**: 508-511
- 29 **De Gregorio MA**, Mainar A, Tejero E, Tobio R, Alfonso E, Pinto I, Fernandez R, Herrera M, Fernandez JA. Acute colorectal obstruction: stent placement for palliative treatment—results of a multicenter study. *Radiology* 1998; **209**: 117-120
- 30 **Baron TH**, Dean PA, Yates MR 3rd, Canon C, Koehler RE. Expandable metal stents for the treatment of colonic obstruction: techniques and outcomes. *Gastrointest Endosc* 1998; **47**: 277-286
- 31 **Gukovsky-Reicher S**, Lin RM, Sial S, Garrett B, Wu D, Lee T, Lee H, Arnell T, Stamos MJ, Eysselein VE. Self-expandable metal stent in palliation of malignant gastrointestinal obstruction: review of the current literature data and 5-year experience at Harbor-UCLA Medical Center. *Medscape General Medicine* 2003; **5**(1). Available from: URL: <http://www.medscape.com/viewarticle/444668>. Posted 01/10/2003
- 32 **Dohmoto M**, Hunerbein M, Schlag PM. Application of rectal stents for palliation of obstructing rectosigmoid cancer. *Surg Endosc* 1997; **11**: 758-761
- 33 **Obayashi M**, Katube T, Shimizu N, Kotani J, Takano Y, Amano R, Yanagawa K, Nishimori T, Sawa Y, Matsumoto T, Arakawa T. Endoscopic placement of metallic stent for colonic stricture resulting from carcinoma located at the splenic flexure. *Dig Endosc* 2002; **14**: 123-127
- 34 **Binkert CA**, Ledermann H, Jost R, Saurenmann P, Decurtins M, Zollikofer CL. Acute colonic obstruction: clinical aspects and cost-effectiveness of preoperative and palliative treatment with self-expanding metallic stents – a preliminary report. *Radiology* 1998; **206**: 199-204
- 35 **Paul Diaz L**, Pinto Pabon I, Fernandez lobato R, Montes Lopez C. Palliative treatment of malignant colorectal strictures with metallic stents. *Cardiovasc Intervent Radiol* 1999; **22**: 29-36
- 36 **Rupp KD**, Dohmoto M, Meffert R, Holzgreve A, Hohlbach G. Cancer of the rectum-palliative endoscopic treatment. *Eur J Surg Oncol* 1995; **21**: 644-647
- 37 **Mergener K**, Kozarek RA. Stenting of the gastrointestinal tract. *Dig Dis* 2002; **20**: 173-181
- 38 **Mauro MA**, Koehler RE, Baron TH. Advances in gastrointestinal intervention: the treatment of gastroduodenal and colorectal obstructions with metallic stents. *Radiology* 2000; **215**: 659-669
- 39 **Tejero E**, Fernandez-Lobato R, Mainar A, Montes C, Pinto I, Fernandez L, Jorge E, Lozano R. Initial results of a new procedure for treatment of malignant obstruction of the left colon. *Dis Colon Rectum* 1997; **40**: 432-436
- 40 **Turegano-Fuentes F**, Echenagusia-Belda A, Simo-Muerza G, Camunez F, Munoz-Jimenez F, Del Valle Hernandez E, Quintans-Rodriguez A. Transanal self-expanding metal stents as an alternative to palliative colostomy in selected patients with malignant obstruction of the left colon. *Br J Surg* 1998; **85**: 232-235
- 41 **Feretis C**, Benakis P, Dimopoulos C, Georgopoulos K, Manouras A, Apostolidis N. Palliation of large-bowel obstruction due to recurrent rectosigmoid tumor using self-expandable endoprostheses. *Endoscopy* 1996; **28**: 319-322
- 42 **Spinelli P**, Dal Fante M, Mancini A. Rectal metal stents for palliation of colorectal malignant stenosis. *Bildgebung* 1993; **60**(Suppl 1): 48-50
- 43 **Mainar A**, Tejero E, Maynar M, Ferral H, Castaneda-Zuniga W. Colorectal obstruction: treatment with metallic stents. *Radiology* 1996; **198**: 761-764
- 44 **Saida Y**, Sumiyama Y, Nagao J, Takase M. Stent endoprosthesis for obstructing colorectal cancers. *Dis Colon Rectum* 1996; **39**: 552-555
- 45 **Choo IW**, Do YS, Suh SW, Chun HK, Choo SW, Park HS, Kang SK, Kim SK. Malignant colonic obstruction: treatment with a flexible covered stent. *Radiology* 1998; **206**: 415-421
- 46 **Adam A**, Morgan R, Ellul J, Mason RC. A new design of the esophageal wallstent endoprosthesis resistant to distal migration. *Am J Roentgenol* 1998; **170**: 1477-1481
- 47 **Chevallier P**, Baque P, Benchimol D, Bernard J, Souci J, Chevallier A, Bourgeon A, Padovani B. Treatment of colorectal obstruction



- with self-expanding metallic stents under fluoroscopic guidance. *J Radiol* 2002; **83**(4 Pt 1): 473-477
- 48 **Spinelli P**, Mancini A. Use of self-expanding metal stents for palliation of rectosigmoid cancer. *Gastrointest Endosc* 2001; **53**: 203-206
- 49 **Lo SK**. Metallic stenting for colorectal obstruction. *Gastrointest Endosc Clin N Am* 1999; **9**: 459-477
- 50 **Coco C**, Cogliandolo S, Riccioni ME, Ciletti S, Marino-Cosentino L, Coppola R, Picciocchi A. Use of a self-expanding stent in the palliation of rectal cancer recurrences. A report of three cases. *Surg Endosc* 2000; **14**: 708-711
- 51 **Camunez F**, Echenagusia A, Simo G, Turegano F, Vazquez J, Barreiro-Meiro I. Malignant colorectal obstruction treated by means of self-expanding metallic stents: effectiveness before surgery and in palliation. *Radiology* 2000; **216**: 492-497
- 52 **Chong LW**, Sun CK, Yang KC. Application of the self-expandable metallic stent for palliation of obstructing rectosigmoid cancer: report of a case. *J Intern Med Taiwan* 2002; **13**: 293-299
- 53 **Baron TH**. Expandable metal stents for the treatment of cancerous obstruction of the gastrointestinal tract. *N Engl J Med* 2001; **344**: 1681-1687
- 54 **Morino M**, Bertello A, Garbarini A, Rozzio G, Repici A. Malignant colonic obstruction managed by endoscopic stent decompression followed by laparoscopic resections. *Surg Endosc* 2002; **16**: 1483-1487
- 55 **McGrath K**. Clinical applications for expandable metal stents in the lumen of the gastrointestinal tract. *MedGenMed* **3**, 2001 [formerly published in *Medscape Gastroenterology eJournal* 3 (3), 2001]. Available at: <http://www.medscape.com/viewarticle/407972>
- 56 **Ely CA**, Arregui ME. The use of enteral stents in colonic and gastric outlet obstruction. *Surg Endosc* 2003; **17**: 89-94
- 57 **Kusayanagi S**, Kaneko K, Yamamura F, Hirakawa M, Miyasaka N, Konishi K, Kurahashi T, Yoshikawa N, Tsunoda A, Kusano M, Mitamura K. Histological findings after placement of a self-expanding stent in rectal carcinoma with complete obstruction - case report. *Hepatogastroenterology* 2002; **49**: 412-415
- 58 **Kang SG**, Jung GS, Cho SG, Kim JG, Oh JH, Song HY, Kim ES. The efficacy of metallic stent placement in the treatment of colorectal obstruction. *Korean J Radiol* 2002; **3**: 79-86
- 59 **Repici A**, Reggio D, De Angelis C, Barletti C, Marchesa P, Musso A, Carucci P, Debernardi W, Falco M, Rizzetto M, Saracco G. Covered metal stents for management of inoperable malignant colorectal strictures. *Gastrointest Endosc* 2000; **52**: 735-740
- 60 **Law WL**, Chu KW, Ho JW, Tung HM, Law SY, Chu KM. Self-expanding metallic stent in the treatment of colonic obstruction caused by advanced malignancies. *Dis Colon Rectum* 2000; **43**: 1522-1527
- 61 **Arnell T**, Stamos MJ, Takahashi P, Ojha S, Sze G, Eysselein V. Colonic stents in colorectal obstruction. *Am Surg* 1998; **64**: 986-988
- 62 **McGregor M**. The Technology Assessment Unit (TAU) of the McGill University Health Centre (MUHC). Should the MUHC approve the use of colorectal stents? Available from: [https://upload.mcgill.ca/tau/stents\\_colorectal\\_Feb\\_2003](https://upload.mcgill.ca/tau/stents_colorectal_Feb_2003)
- 63 **Zollikofer CL**, Jost R, Schoch E, Decurtins M. Gastrointestinal stenting. *Eur Radiol* 2000; **10**: 329-341
- 64 **Osman HS**, Rashid HI, Sathananthan N, Parker NC. The cost effectiveness of self-expanding metal stents in the management of malignant left-sided large bowel obstruction. *Colorectal Disease* 2000; **2**: 233-237

Edited by Wang XL Proofread by Zhu LH

• CASE REPORT •

# Successful treatment with rifampin for fulminant antibiotics-associated colitis in a patient with non-Hodgkin's lymphoma

Kenichi Nomura, Yosuke Matsumoto, Naohisa Yoshida, Sawako Taji, Naoki Wakabayashi, Shoji Mitsufuji, Shigeo Horiike, Masuji Morita, Takeshi Okanoue, Masafumi Taniwaki

**Kenichi Nomura, Yosuke Matsumoto, Shigeo Horiike**, Molecular Hematology and Oncology, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto, Japan

**Naohisa Yoshida, Sawako Taji, Naoki Wakabayashi, Shoji Mitsufuji, Takeshi Okanoue**, Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto, Japan

**Masuji Morita**, School of Nursing, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto, Japan

**Masafumi Taniwaki**, Clinical Molecular Genetics and Laboratory Medicine, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto, Japan

**Correspondence to:** Kenichi Nomura, M.D. Ph.D., Molecular Hematology and Oncology, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto, 602-0841, Japan. nomuken@sun.kpu-m.ac.jp  
**Telephone:** +81-75-251-5521 **Fax:** +81-75-251-0710

**Received:** 2003-11-12 **Accepted:** 2003-12-16

## Abstract

A 74-year-old man was admitted to the hospital because of chemotherapy for relapsed non-Hodgkin's lymphoma (NHL). The patient became febrile and experienced diarrhea after chemotherapy. Although ceftazidime and amikacin sulfate were administered as empiric therapy, diarrhea was continued. After several days, stool cytotoxin assay for *Clostridium difficile* (*C. difficile*) was positive and he was diagnosed as having antibiotics-associated colitis (AAC). Although antibiotics were discontinued and both oral vancomycin and metronidazole were administered, disease was not improved. To rule out the presence of an additional cause of diarrhea, colon fiberoptic examination was performed. It revealed multiple deep ulcerative lesions at right side colon, surface erosive and minute erosive lesions in all continuous colon. Pseudomembranes were not seen. These findings are compatible with AAC without pseudomembranes. There are no reports that the rifampin is effective on refractory AAC. However, we administered oral rifampin for the current patient. The reasons are 1) conventional antibiotics were not effective, 2) rifampin has excellent *in vitro* activity against *C. difficile*, and 3) the efficacy of rifampin on relapsing colitis due to *C. difficile* is established. After administration of rifampin, fever alleviated and diarrhea was improved. Because AAC may result in significant mortality, patients with refractory or fulminant AAC should be treated with oral rifampin from outset.

Nomura K, Matsumoto Y, Yoshida N, Taji S, Wakabayashi N, Mitsufuji S, Horiike S, Morita M, Okanoue T, Taniwaki M. Successful treatment with rifampin for fulminant antibiotics-associated colitis in a patient with non-Hodgkin's lymphoma. *World J Gastroenterol* 2004; 10(5): 765-766  
<http://www.wjgnet.com/1007-9327/10/765.asp>

## INTRODUCTION

Antibiotics-associated colitis (AAC) due to *Clostridium difficile*

(*C. difficile*) is a nosocomial infection that may result in significant morbidity and mortality. AAC is capable of causing toxigenic colitis in susceptible patients such as those receiving chemotherapy. Approximately, 50% cases having endoscopic evidence of pseudomembranous colitis revealed exudative and punctate raised plaques with slip areas. The remainder had milder degrees of colitis without pseudomembranes<sup>[1,2]</sup>. The clinical features of AAC without pseudomembranes are thought to be similar to those seen with pseudomembranes colitis, but of less severity<sup>[3,4]</sup>. Patients with mild colitis are improved only by discontinuation of antibiotics. However, most patients are now treated with vancomycin (VCM) or metronidazole capable of eradicating *C. difficile* infection because of high mortality from AAC. The typical response to VCM therapy is improvement in diarrhea within a few days.

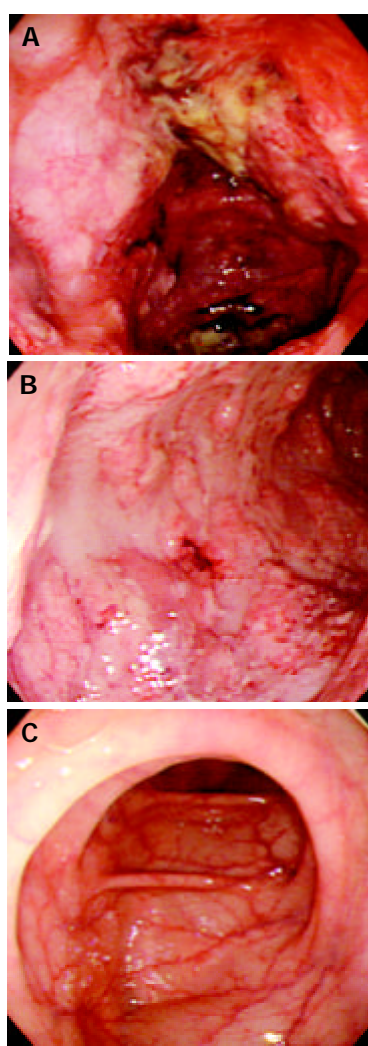
The recommendation is not established for patients with fulminant AAC. Colectomy may be life-saving in patients with severe AAC who fail to respond to antibiotic therapy. However, the surgical mortality is approximately 50%<sup>[5]</sup>.

We described a refractory patient having AAC without pseudomembranes. This patient was refractory to metronidazole and VCM. We administered oral rifampin for the current patient and he gained improvement. This is the first report that rifampin is effective for refractory or fulminant AAC.

## CASE REPORT

A 74-year-old man was admitted to our hospital in May 2002 because of chemotherapy for relapsed non-Hodgkin's lymphoma. He presented a huge mass of NHL at lumber. He was treated with 3 courses of VeMP combination chemotherapy<sup>[6]</sup>. After the first VeMP chemotherapy, he complained of diarrhea. Fosfomycin calcium (FOM) was administered and diarrhea was stopped. There was no side effect during the second course of VeMP chemotherapy. During the third chemotherapy, he became febrile without diarrhea, we administered ceftazidime (CAZ) and amikacin sulfate (AMK) as empiric therapy. Fever alleviated immediately. Considering the high frequencies of relapse in patients treated with VeMP, we stopped VeMP therapy and started CHOP therapy (cyclophosphamide, adriamycin, vincristine, and prednisolone) on 6 August. Because the patient became febrile and experienced diarrhea from d 4, we administered lactomin. On d 8, the patient developed massive watery diarrhea (over 1 500 mL/d) with exacerbation of fever, respiratory distress and marked hypovolemia. Ultrasound examination revealed massive ascites and pleural effusion. Although bolus methylprednisolone was administered promptly and CAZ and AMK as empiric therapy, the patient remained febrile and watery green diarrhea was not controlled. Salmonella, Shigella, campylobacter, cryptosporidium, fecal viruses and parasites were negative in repeated stool cultures. Because *C. difficile* toxin was detected using an enzyme immunoassay (Meridian Diagnostics, Inc., Cincinnati, OH, USA) in stools on day 13, the patient was diagnosed as having AAC. Although CAZ and AMK were stopped and oral VCM and metronidazole 250 mg

daily were started concomitantly, symptom did not improve. Colonoscopic examination revealed multiple deep ulcerative lesions at right side colon, surface erosive and minute erosive lesions in all continuous colon. Pseudomembranes were not seen. Biopsy reveals an outpouring of fibrin, mucus, and inflammatory cells from a microulceration of the surface epithelium. These findings are compatible with AAC without pseudomembranes. Because VCM and metronidazole were not effective, the enteral use of rifampin 600 mg twice daily was started on d 23. In the following several days, fever alleviated and diarrhea was improved (Figure 1).



**Figure 1** A: Multiple deep ulcerative lesions at right side colon, B: Surface erosive and minute erosive lesions in all continuous colon, C: After administration of rifampin.

## DISCUSSION

We described a patient having refractory AAC without pseudomembranes. The current patient presented severe

watery diarrhea, malaise, fever, leukocytosis and dehydration. Administration of VCM and metronidazole was not effective. Some treatment regimens as the second therapy for refractory AAC have been tried, but none has been completely effective in randomized clinical trials. Although there have been some reports of the efficacy of oral teicoplanin for the treatment of antibiotics-associated colitis<sup>[7,8]</sup>, oral teicoplanin is not available in Japan. Colectomy was not required, because the current patient had a substantial comorbid disease and was extremely ill.

Rifampin had excellent *in vitro* activity against *C. difficile* with minimum inhibitory concentrations averaging  $<0.2 \mu\text{g/mL}$ <sup>[9,10]</sup> and the efficacy of rifampin on relapsing colitis due to *C. difficile* was established<sup>[11]</sup>. Thus, we administered rifampin 600 mg twice daily. Diarrhea and abdominal pain resolved promptly. Although there are no reports that rifampin is a useful agent for refractory and severe AAC, patients should be treated with oral rifampin from the outset.

## REFERENCES

- 1 **Lishman AH**, Al-Jumaili IJ, Record CO. Spectrum of antibiotic-associated diarrhoea. *Gut* 1981; **22**: 34-37
- 2 **Bergstein JM**, Kramer A, Wittman DH, Aprahamian C, Quebbeman EJ. Pseudomembranous colitis: how useful is endoscopy? *Surg Endosc* 1990; **4**: 217-219
- 3 **Totten MA**, Gregg JA, Fremont-Smith P, Legg M. Clinical and pathological spectrum of antibiotic-associated colitis. *Am J Gastroenterol* 1978; **69**(3 Pt 1): 311-319
- 4 **Gerding DN**, Olson MM, Peterson LR, Teasley DG, Gebhard RL, Schwartz ML, Lee JT Jr. Clostridium difficile-associated diarrhea and colitis in adults. A prospective case-controlled epidemiologic study. *Arch Intern Med* 1986; **146**: 95-100
- 5 **Klingler PJ**, Metzger PP, Seelig MH, Pettit PD, Knudsen JM, Alvarez SA. Clostridium difficile infection: risk factors, medical and surgical management. *Dig Dis* 2000; **18**: 147-160
- 6 **Santini G**, Contu A, Porcellini A, Chisesi T, Coser P, Congiu AM, Morandi S, Manna A, Schintu GM, Quanini R, Rancan L, Miglio L, Damasio E, Rizzoli V. Mitoxantrone alone or in combination chemotherapy (VeMP) as second-line treatment in relapsed or refractory poor-prognosis non-Hodgkin's lymphoma. A report of the Non-Hodgkin's Lymphoma Co-operative Study Group (NHLCSG). *Haematologica* 1991; **76**: 485-490
- 7 **de Lalla F**, Privitera G, Rinaldi E, Ortisi G, Santoro D, Rizzardini G. Treatment of Clostridium difficile-associated disease with teicoplanin. *Antimicrob Agents Chemother* 1989; **33**: 1125-1127
- 8 **Wenisch C**, Parschalk B, Hasenhundl M, Hirschl AM, Graninger W. Comparison of vancomycin, teicoplanin, metronidazole, and fusidic acid for the treatment of Clostridium difficile-associated diarrhea. *Clin Infect Dis* 1996; **22**: 813-818
- 9 **O'Connor RP**, Silva J Jr, Fekety R. Rifampicin and antibiotic-associated colitis. *Lancet* 1981; **1**: 499
- 10 **Ripa S**, Mignini F, Prenna M, Falcioni E. *In vitro* antibacterial activity of rifaximin against Clostridium difficile, Campylobacter jejuni and Yersinia spp. *Drugs Exp Clin Res* 1987; **13**: 483-488
- 11 **Buggy BP**, Fekety R, Silva J Jr. Therapy of relapsing Clostridium difficile-associated diarrhea and colitis with the combination of vancomycin and rifampin. *J Clin Gastroenterol* 1987; **9**: 155-159

Edited by Wang XL Proofread by Zhu LH

• CASE REPORT •

## Recurrent inflammatory fibroid polyp of cardia: A case report

Krzysztof Zinkiewicz, Witold Zgodziński, Andrzej D<sup>1</sup> browski, Justyna Szumi<sup>3</sup> o, Grzegorz Ąwik, Grzegorz Wallner

**Krzysztof Zinkiewicz, Witold Zgodziński, Andrzej D<sup>1</sup> browski, Grzegorz Ąwik, Grzegorz Wallner**, 2<sup>nd</sup> Department of General Surgery, Skubiszewski Medical University of Lublin, Staszica 16, 20-081, Lublin, Poland

**Justyna Szumi<sup>3</sup> o**, Department of Human Pathology, Skubiszewski Medical University of Lublin, Jaczewskiego 8, 20-950, Lublin, Poland

**Correspondence to:** Krzysztof Zinkiewicz, MD, 2<sup>nd</sup> Department of General Surgery, Skubiszewski Medical University of Lublin, Staszica 16, 20-081, Lublin, Poland. kzinek@yahoo.com

**Telephone:** +4881-53-241-27 **Fax:** +4881-53-288-10

**Received:** 2003-11-04 **Accepted:** 2003-12-24

### Abstract

Inflammatory fibroid polyp is one of the chronic inflammatory diseases in the digestive tract, which often mimics the submucosal tumor. Precise diagnosis is possible after removal of the detected lesion. Endoscopic removal is recommended as a safe and efficient method of the treatment. In this report the authors present a case of inflammatory fibroid polyp located in the cardia, which has been removed endoscopically. Twelve months later, recurrence of the lesion was noted and the patient was referred to surgical resection.

Zinkiewicz K, Zgodziński W, D<sup>1</sup> browski A, Szumi<sup>3</sup> o J, Ąwik G, Wallner G. Recurrent inflammatory fibroid polyp of cardia: A case report. *World J Gastroenterol* 2004; 10(5): 767-768  
<http://www.wjgnet.com/1007-9327/10/767.asp>

### INTRODUCTION

Inflammatory fibroid polyp (IFP) in the digestive tract is one of the chronic inflammatory diseases, which most often is located in the stomach. Precise diagnosis of IFP remains difficult because available methods such as barium swallow or even endoscopy with endoscopic ultrasonography (EUS) provide only nonspecific, insufficient information. Some cases of IFP can macroscopically mimic gastric cancer<sup>[1,2]</sup>. Hence, it is most important to exclude malignancy by histology on the detected lesion. In the case of submucosal lesions, standard biopsies are insufficient in obtaining adequate diagnostic tissue. Then endoscopic tumor excision or polypectomy is recommended. However the curative significance of endoscopic treatment in patients with IFP is still under discussion. We would like to report a rare case of IFP, localized within the esophago-gastric junction, which recurred 12 mo after endoscopic removal.

### CASE REPORT

A 48-year-old male with symptoms of dysphagia for solids and epigastric pain of moderate intensity, lasting for 3 weeks, was admitted to the hospital. No weight loss was noted. The patient had no history of any other diseases including parasites and allergies. No abnormalities were found upon physical examination and laboratory results (hematology, serum biochemistry, urine analysis) were within normal ranges. Gastrofiberoscopy revealed an elevated, round, polypoid tumor, 15×20 mm with small ulceration in the center filled

with necrotic tissue (Figures 1, 2). The tumor was located exactly within the esophagogastric junction and significantly reduced the lumen of the cardia. The surface of the lesion was covered with hypertrophic mucosus. On retroflexion, the tumor was entirely within the confines of the stomach, suggesting the possibility of complete endoscopic excision (Figure 3). EUS showed a low echoic but rather homogenous tumor which did not invade the muscular layer. No other pathologies in the upper part of gastrointestinal tract were found. Endoscopic biopsies showed normal mucosa and attention was directed to endoscopic removal. Macroscopically radical endoscopic excision of the tumor, completed with argon plasma coagulation of its basement was performed under general anesthesia, and removed lesion was investigated by pathologists. Histological examination revealed a superficially ulcerated and well-circumscribed but nonencapsulated submucosal tumor, which extended partly into the cardiac mucosa. The tumor was composed of fibrous tissue with abundant small blood vessels and numerous eosinophils admixed with plasma cells and lymphocytes (Figure 4A,B). The lymphoid aggregates were also occasionally seen within the tumor and in adjacent tissues (Figure 4B). The inflammatory fibroid polyp was diagnosed. One day after the endoscopic procedure, the patient was discharged from the hospital with no postoperative complications noted.

Twelve months after the treatment, the patient was admitted to the hospital with the same symptoms as previously. Gastrofiberoscopy revealed the presence of the tumor in the cardia with an endoscopic appearance mostly as described above. The recurrent tumor was larger, 25×39 mm as shown on barium contrast radiography, and was more proximally located. In light of these findings the patient was referred to surgical treatment. Proximal gastrectomy with local lymphadenectomy, via laparotomy and distal esophagectomy via right thoracotomy with esophago-gastrostomy within the posterior mediastine were performed. Histological appearance of the recurrent tumor was identical with the first one. Postoperative course was uneventful and the patient was discharged 15 d after surgery. No significant changes within 4-mo follow-up were observed. Follow-up fiberoscopy performed 3 mo after the surgery showed no pathological signs within the esophago-gastrostomy.

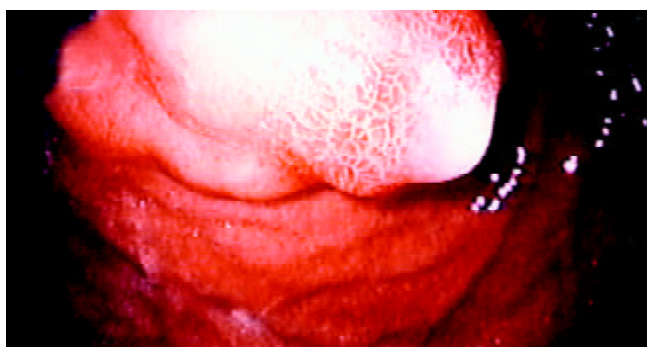


**Figure 1** Endoscopic view of inflammatory fibroid polyp (IFP) located in cardia. An elevated, round, polypoid tumor can be seen.

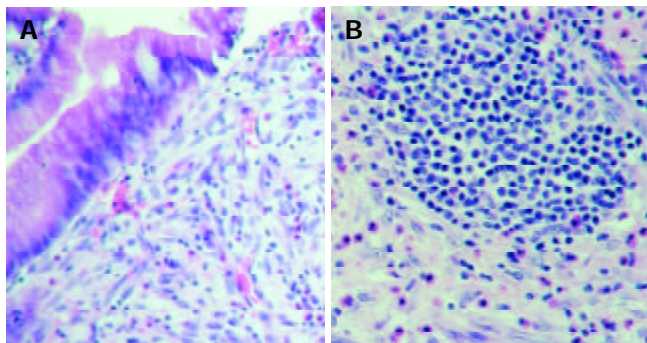




**Figure 2** Inflammatory fibroid polyp of cardia. A small ulceration can be seen in the center of the lesion filled with necrotic tissue.



**Figure 3** Retroflexion of endoscope. The tumor is indented into the stomach.



**Figure 4** Histologic appearance of primary inflammatory fibroid polyp of cardia. A superficially ulcerated tumor can be observed involving cardiac mucosa and submucosa, and consists of fibrovascular tissue as well as numerous eosinophils admixed with plasma cells and lymphocytes. (HE; A: magn.  $\times 100$ ; B: magn.  $\times 200$ ).

## DISCUSSION

In 1953 Helwig and Rainer proposed the term of inflammatory fibroid polyp for eosinophilic granuloma of the stomach which presently is generally accepted<sup>[3]</sup>. IFP arises from submucosa of the gastrointestinal tract. It consists of loose connective tissue with a rich vasculature and abundant fibrous component. Usually the lesion was sessile or polypoid with ulceration of the overlying mucosa<sup>[4]</sup>. IFP was mainly located in the pyloric region of the stomach, less frequent in the ileum, and only occasionally in the colon or esophagus<sup>[4-6]</sup>. Small lesion is usually asymptomatic until pyloric stenosis or small bowel obstruction occurs. In the case described in the present report, a relatively small IFP was detected due to symptoms of

dysphagia. Its localization in the esophago-gastric junction has not been reported previously. Although IFP was nonneoplastic in nature<sup>[4,6]</sup>, its cause remains unclear. Fibroblastic<sup>[4]</sup> or vascular origin of IFP<sup>[7]</sup> has been considered. Eosinophilic infiltration which sometimes may occur as a submucosal tumor was also related to parasitic infections such as gastric anisakiasis<sup>[8,9]</sup>.

However, endoscopists should always suspect submucosal mesenchymal tumors of being gastrointestinal stromal tumor (GIST), leiomyoma or leiomyosarcoma especially when tumor macroscopically mimics a malignant lesion. Biopsy specimens using standard forceps may not be adequate for histological diagnosis when tumor is covered with normal mucosa. Then endoscopic excision/polypectomy preceded with EUS should be the best diagnostic method. There were some reports concerning the curative role of endoscopic removal of IFP<sup>[10-12]</sup>. However, our results indicate the possibility of local recurrence of IFP after endoscopic treatment. Thus we recommend endoscopic removal as the most valuable diagnostic method for providing the specimens to accurate histological assessment. It may be reserved as the curative procedure in elderly and high-risk patients as has been proposed previously<sup>[11]</sup> and should be repeated if necessary. Elevation of the tumor after submucosal saline or xylocaine injection can be helpful in making decision of endoscopic removal, and may indicate the possibility of complete, radical dissection. In other cases, minimal invasive surgery, *i.e.* laparoscopic resection, may be considered. To date only a single report has been available about recurrent eosinophilic granuloma of the ileum in a 2-year-old child<sup>[13]</sup>.

## REFERENCES

- 1 **Yoh H**, Natsugoe S, Ohsako T, Yamada K, Suenaga T, Hokita S, Ohi H, Nishimata Y, Nishimata H, Aikou T. Eosinophilic granuloma of the stomach mimicking gastric cancer, report of a case. *Hepatogastroenterology* 2001; **48**: 606-608
- 2 **Premaratna R**, Saparamadu A, Samarasekera DN, Warren BF, Jewell DP, de Silva HJ. Eosinophilic granulomatous vasculitis mimicking a gastric neoplasm. *Histopathology* 1999; **35**: 479-481
- 3 **Helwig EB**, Ranier A. Inflammatory fibroid polyps of the stomach. *Surg Gynecol Obstet* 1953; **96**: 355-367
- 4 **Johnstone JM**, Morson BC. Inflammatory fibroid polyp of the gastrointestinal tract. *Histopathology* 1978; **2**: 349-361
- 5 **Samter TG**, Alstott DF, Kurlander GJ. Inflammatory fibroid polyps of the gastrointestinal tract. A report of 3 cases, 2 occurring in children. *Am J Clin Pathol* 1966; **45**: 420-436
- 6 **Shimer GR**, Helwig EB. Inflammatory fibroid polyps of the intestine. *Am J Clin Pathol* 1984; **81**: 708-714
- 7 **Pack GT**. Unusual tumors of the stomach. *Ann N Y Acad Sci* 1964; **114**: 985-1011
- 8 **Sakai K**, Ohtani A, Muta H, Tominaga K, Chijiwa Y, Hiroshige K, Fujishima H, Ohkubo A, Misawa T, Nawata H. Endoscopic ultrasonography findings in acute gastric anisakiasis. *Am J Gastroenterol* 1992; **87**: 1618-1623
- 9 **Takeuchi K**, Hanai H, Iida T, Suzuki S, Isobe S. A bleeding gastric ulcer on a vanishing tumor caused by anisakiasis. *Gastrointest Endosc* 2000; **52**: 549-551
- 10 **Tada S**, Iida M, Yao T, Matsui T, Kuwano Y, Hasuda S, Fujishima M. Endoscopic removal of inflammatory fibroid polyps of the stomach. *Am J Gastroenterol* 1991; **86**: 1247-1250
- 11 **Eugene C**, Penalba C, Gompel H, Bergue A, Felsenheld C, Fingerhut A, Quevauvilliers J. Gastric eosinophilic granuloma: value of endoscopic polypectomy. Apropos of 2 cases. *Sem Hop* 1983; **59**: 2249-2250
- 12 **Matsushita M**, Hajiro K, Okazaki K, Takakuwa H. Endoscopic features of gastric inflammatory fibroid polyps. *Am J Gastroenterol* 1996; **91**: 1595-1598
- 13 **McGreevy P**, Doberneck RC, McLeay JM, Miller FA. Recurrent eosinophilic infiltrate (granuloma) of the ileum causing intussusception in a two-year-old child. *Surgery* 1967; **61**: 280-284

# Endoscopic retrieval of multiple fragmented gastric bamboo chopsticks by using a flexible overtube

Jia-Jang Chang, Cho-Li Yen

**Jia-Jang Chang, Cho-Li Yen**, Department of Hepatogastroenterology, Chang Gung Memorial Hospital, Keelung, Taiwan, China

**Correspondence to:** Cho-Li Yen, M.D. Department of Hepatogastroenterology, Chang Gung Memorial Hospital, Keelung, 222, Mai Chin Road, Keelung, Taiwan, 204, China. g15539@cgmh.org.tw

**Telephone:** +886-2-24313131~2627 **Fax:** +886-2-24335342

**Received:** 2003-10-20 **Accepted:** 2003-12-24

## Abstract

This is a rare case of a patient with mental disorder, who ingested nineteen pieces of fragmented bamboo chopsticks. We managed the multiple gastric foreign bodies with a sclerotherapy overtube, and these multiple fragmented bamboo chopsticks were retrieved successfully using the endoscopic method. There were only multiple erosions with hemorrhage over the mucosa of fundus and body of stomach, no fragments adhered or perforated through the gastric wall. The mucosa of esophagus was intact. The patient tolerated the procedure well and without any major complications. Multiple sharp elongated gastric foreign bodies can be successfully and safely retrieved by using protective sheath of oropharynx without assistance with laparoscopy or surgical intervention. This renders an option for the endoscopists to manage multiple elongated gastric foreign bodies.

Chang JJ, Yen CL. Endoscopic retrieval of multiple fragmented gastric bamboo chopsticks by using a flexible overtube. *World J Gastroenterol* 2004; 10(5): 769-770

<http://www.wjgnet.com/1007-9327/10/769.asp>

## INTRODUCTION

Foreign body ingestion is a common problem in the emergency department. Most ingestions may be accidental, but may also be a result of contributory factors as mental disorder, bulimia, alcohol consumption, and prison inmates<sup>[1]</sup>. When foreign bodies are ingested, they will usually pass spontaneously through the whole alimentary tract and out to the feces. The risk of perforation of gastrointestinal tract is only 1%<sup>[2]</sup>. Ten to twenty percent of the objects will have to be removed endoscopically, about 1% will require surgery<sup>[1]</sup>. However, sharp and pointed foreign bodies, as well as elongated materials in the stomach, can be very challenging and difficult to manage by endoscopy. Long and sharp foreign bodies should be removed immediately before they pass from the stomach to the intestine, as 15% to 35% of them will cause intestinal perforation<sup>[3]</sup>. Elongated materials such as toothbrushes, toothpicks, and bones are the most common foreign bodies in the stomach that require surgery for their removal<sup>[4,5]</sup>. Here, we report a rare case of a patient with mental disorder, who ingested nineteen pieces of fragmented chopsticks. The problem was treated successfully using the endoscopic method.

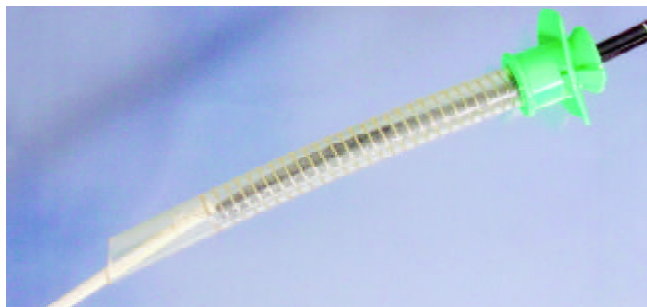
## CASE REPORT

The case of a 24 year-old man had a history of substance

dependence. He had used amphetamine for years, intermittently in the first three years and thereafter nearly everyday. As he developed a physical tolerance to amphetamine, he then turned to alcohol abuse. He was admitted to a psychiatry ward because of his drug and alcohol addictions. Two weeks after he was discharged from the psychiatry ward, he stayed at home doing nothing and continued alcohol abuse. His mother described his behavior as being mood swinging from depression to mania. He suffered from insomnia, having had hot temper, restlessness, to being bed-ridden and mental disturbed. He was forced by his mother to admit to a daytime psychiatric ward again for substance dependence. After admission, he committed to suicide by swallowing fragmented bamboo chopsticks. His roommate found that he swallowed small pieces of chopsticks and complained of epigastric pain 4 d after admission. The nurse was notified and called the doctor to check him up. As the patient stated that he began to swallow in fragmented bamboo chopsticks piece by piece at meals 4 d before. On physical examination, his abdomen was found to be soft, but he had tenderness over the epigastric area, and he produced a normally active bowel sound. A plain abdomen radiography did not show foreign body. An upper gastrointestinal panendoscopy was performed on March 14, 2000 to ascertain if he had indeed ingested chopsticks. In the left lateral decubitus position, the patient was given local anesthetic. Afterward, he was given intravenous hyoscine-N-butylbromide (20 mg) and sedated with intravenous midazolam (5 mg). There were food residues mixed with plenty of fragmented bamboo chopsticks with one or two sharp ends in the fundus and body of the stomach (Figure 1). We placed a "flexible overtube" (Sumitomo®, Japan) through the mouth to esophagus and used Dormia basket to grasp the blunt end of fragmented bamboo chopsticks out of the overtube. The blunt ends were grasped tightly at the uppermost part, and withdrawn through the esophago-cardiac junction without any difficulty. Small fragments could be easily and repeatedly passed through the esophagus into the channel of the overtube, whereas the larger fragments (more than 10 cm long) stuck at the oropharynx where the angle is almost rectangular (Figure 2). To remove the long fragments we withdrew the gastroscope together with the overtube. Since five of the fragments were more than 10 cm, we repeated this operation 5 times. Sumitomo® overtube which is quite flexible and elastic could be passed through the oropharynx to the esophagus repeatedly and easily without causing injury to the esophagus. The fragmented chopsticks were mixed with food debris, making blunt ends difficult to be found and grasped. It took 2 h to accomplish the procedure. In all, a total of nineteen fragmented bamboo chopsticks were retrieved (Figure 3). After removal of the fragmented bamboo chopsticks, the esophagus and stomach were thoroughly examined. There were multiple erosions and hemorrhage spots over the mucosa of the fundus and the body, fortunately no fragment pieced through the gastric wall. The mucosa of esophagus was intact. The patient was observed for 2 d, he had no complaint except mild epigastric pain that could be controlled by antacids. He was referred for psychiatric evaluation and discharged uneventfully.



**Figure 1** Plenty of fragmented bamboo chopsticks mixed with food debris in fundus and body of stomach.



**Figure 2** Impaction of a large piece of fragmented bamboo chopsticks (more than 10 cm long) impacted in channel of Sumitomo sclerotherapy overtube (Simulate picture).



**Figure 3** Retrieval of nineteen fragmented bamboo chopsticks.

## DISCUSSION

About 80% to 90% of small indigestible objects that entered the stomach could eventually pass through the intestinal tract. However, sharp foreign bodies might lodge in the esophagus and lead to esophageal perforation, retroesophageal abscess, mediastinitis, and esophagoaortic fistulae<sup>[4]</sup>. It was very difficult to remove sharp and pointed foreign bodies, as well as elongated objects in the gastrointestinal tract by endoscopic management and they have become a technical challenge<sup>[5]</sup>. Objects that are longer than six centimeters are difficult to pass through the duodenal sweep. If the objects are greater than two centimeter in diameter, they may not pass through the pylorus. Ingested objects longer than six centimeters in children or 13 centimeters in adults should be promptly removed endoscopically due to the high incidence of penetration and entrapment in the bowel<sup>[6]</sup>. Up to 15% to 35% of sharp and pointed foreign bodies ingested would penetrate the wall of the gastrointestinal tract<sup>[3]</sup>. Indicating that when a sharp or pointed foreign body is found in the stomach or duodenum during an endoscopic evaluation, emergent endoscopic removal of the foreign body is mandatory, even if the patient is asymptomatic.

Adults ingesting pointed objects are mostly prisoners or psychiatric patients, and carried a higher complication rate and surgical rate than when that of accidental ingestion. In the review of Gracia *et al.*, there were 22 deliberate ingestors, five of whom had complications of perforation. There was also a high rate of endoscopic failure, up to 80%<sup>[7]</sup>. Bamboo chopsticks are commonly used in China and Far Eastern Asia. In this present case, the young psychiatric patient swallowed fragmented bamboo chopsticks to attract attention of others.

We have tried using the Dormia basket to hold the blunt ends of the fragments and remove them piece by piece, but we failed to remove them because the long bamboo chopsticks would get trapped at the oropharynx. Sometimes the grip of the Dormia basket would be lost when it passed the oropharynx. The endoscopy overtube technique is especially useful in extracting multiple foreign bodies at one time. It could facilitate rapid reinsertion of the endoscope after each retrieval and protect the esophageal mucosa as well as cricopharyngeus muscles from injury<sup>[8,9]</sup>. Werth *et al.* used a protective Terblanche sclerotherapy overtube to remove multiple gastric foreign bodies<sup>[10]</sup>. Yong *et al.* removed a dinner fork from the stomach by using a double snare method to align the axis of the objects and facilitate its withdrawal<sup>[11]</sup>. Wishner *et al.* recommended laparoscopy assisted removal via gastrostomy to remove a swallowed toothbrush<sup>[12]</sup>.

In this case, there were multiple fragmented bamboo chopsticks with various lengths. They measured up to 10 cm each with a sharp broken end. The long chopstick could only be retrieved along with the overtube, because the long chopstick and Dormia Basket could have entrapped in the inner channel of the overtube at the oropharynx level. As a result, repeated intubations of the overtube were necessary. Fortunately, these gastric foreign bodies were successfully and safely retrieved without assistance with laparoscopy or surgical intervention. Therefore, we recommend using repeated insertion of Sumitomo® sclerotherapy overtube, which is flexible and easy to pass through the oropharynx, to extract the ingested sharp foreign bodies. The maneuver is safe although time-consuming. This renders an option for endoscopists to manage multiple elongated and pointed gastric foreign bodies.

## REFERENCES

- 1 Webb WA. Management of foreign bodies of the upper gastrointestinal tract. *Gastrointest Endosc* 1995; **41**: 39-51
- 2 Carp L. Foreign bodies in the intestine. *Ann Surg* 1927; **85**: 575-591
- 3 Rosch W, Classen M. Fibroendoscopic foreign body removal from the upper gastrointestinal tract. *Endoscopy* 1972; **4**: 193-197
- 4 Nandi P, Ong GB. Foreign bodies in the esophagus: review of 2394 cases. *Br J Surg* 1978; **65**: 5-9
- 5 Stack LB, Munter DW. Foreign bodies in the gastrointestinal tract. *Emerg Med Clin North Am* 1996; **14**: 493-521
- 6 Brady PG. Esophageal foreign bodies. *Gastroenterol Clin North Am* 1991; **20**: 691-701
- 7 Gracia C, Frey CF, Bodai BI. Diagnosis and management of ingested foreign body: a ten-year experience. *Am Emerg Med* 1984; **13**: 30-34
- 8 Rogers BH, Kot C, Meiri S. An overtube for the flexible fibroptic esophagogastroduodenoscopy. *Gastrointest Endosc* 1982; **28**: 256
- 9 Spurling TJ, Zaloga GP, Richter JE. Fiberendoscopic removal of a gastric foreign body with overtube technique. *Gastrointest Endosc* 1983; **29**: 226-227
- 10 Werth RW, Edwards C, Jennings WC. A safe and quick method for endoscopic retrieval of multiple gastric foreign bodies using a protective sheath. *Surg Gynecol Obstetrics* 1990; **171**: 419-420
- 11 Yong PTL, Teh CH, Look M, Wee SB, Tan JCH, Chew SP, Low CH. Removal of a dinner fork from the stomach by double snare endoscopic extraction. *Hong Kong Med J* 2000; **6**: 319-12
- 12 Wishner JD, Rogers AM. Laparoscopic removal of a swallowed toothbrush. *Surgical Endoscopy* 1997; **11**: 472-473