

World Journal of Gastroenterology®

Volume 13 Number 30
August 14, 2007



National Journal Award
2005



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

World Journal of Gastroenterology

www.wjgnet.com

Volume 13

Number 30

Aug 14

2007



ISSN 1007-9327
CN 14-1219/R



WJG

World Journal of Gastroenterology®

Indexed and Abstracted in:

Current Contents®/Clinical Medicine, Science
Citation Index Expanded (also known as
SciSearch®) and Journal Citation Reports/Science
Edition, *Index Medicus*, MEDLINE and PubMed,
Chemical Abstracts, EMBASE/Excerpta Medica,
Abstracts Journals, *Nature Clinical Practice
Gastroenterology and Hepatology*, CAB Abstracts
and Global Health.
ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

**Volume 13 Number 30
August 14, 2007**

World J Gastroenterol
2007 August 14; 13(30): 4035-4160

Online Submissions

wjg.wjgnet.com
www.wjgnet.com

Printed on Acid-free Paper
世界胃肠病学杂志

A Weekly Journal of Gastroenterology and Hepatology



National Journal Award
2005

World Journal of Gastroenterology[®]

Weekly Established in October 1995

Volume 13 Number 30
August 14, 2007



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NAME OF JOURNAL
World Journal of Gastroenterology

RESPONSIBLE INSTITUTION
Department of Science and Technology
of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center
for Digestive Diseases, Taiyuan 77,
Shuangta Xijie, Taiyuan 030001, Shanxi
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Gastroenterology*, 77 Shuangta Xijie,
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PUBLISHING
Editorial Department of *World Journal
of Gastroenterology*, 77 Shuangta Xijie,
Taiyuan 030001,
Shanxi Province, China
Telephone: +86-351-4078656
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PRINTING
Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR
Beijing Bureau for Distribution of
Newspapers and Journals
(Code No. 82-261)
China International Book Trading
Corporation PO Box 399, Beijing,
China (Code No. M4481)

PUBLICATION DATE
August 14, 2007

EDITOR-IN-CHIEF
Lian-Sheng Ma, Taiyuan

SUBSCRIPTION
RMB 50 Yuan for each issue, RMB 2400
Yuan for one year

CSSN
ISSN 1007-9327
CN 14-1219/R

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Enteric glial cells and their role in gastrointestinal motor abnormalities: Introducing the neuro-gliopathies

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Received: 2007-04-07 Accepted: 2007-05-12

Abstract

The role of enteric glial cells has somewhat changed from that of mere mechanical support elements, gluing together the various components of the enteric nervous system, to that of active participants in the complex interrelationships of the gut motor and inflammatory events. Due to their multiple functions, spanning from supporting elements in the myenteric plexuses to neurotransmitters, to neuronal homeostasis, to antigen presenting cells, this cell population has probably more intriguing abilities than previously thought. Recently, some evidence has been accumulating that shows how these cells may be involved in the pathophysiological aspects of some diseases. This review will deal with the properties of the enteric glial cells more strictly related to gastrointestinal motor function and the human pathological conditions in which these cells may play a role, suggesting the possibility of enteric neuro-gliopathies.

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Key words: Enteric glia; Glial cells; Gastrointestinal motility

Bassotti G, Villanacci V, Fisogni S, Rossi E, Baronio P, Clerici C, Maurer CA, Cathomas G, Antonelli E. Enteric glial cells and their role in gastrointestinal motor abnormalities: Introducing the neuro-gliopathies. *World J Gastroenterol* 2007; 13(30): 4035-4041

<http://www.wjgnet.com/1007-9327/13/4035.asp>

INTRODUCTION

The enteric nervous system (ENS) is organized in a complex structure that controls motility, blood flow, uptake of nutrients, secretion, immunological and inflammatory processes in the gut^[1]. Two main cell populations are represented in the ENS, neurons and enteric glial cells (EGC), the latter being much more abundant (up to fourfold) than neurons^[2,3] (Figure 1). In humans, the ENS is subdivided into several plexuses (subserous, longitudinal muscle, myenteric, circular muscle, deep muscular, muscularis mucosae, and mucosal)^[4]. Ganglionated plexuses are present in the submucosa (Meissner's and Henle's plexuses) and in the septum between the circular and longitudinal layers of the muscularis propria (Auerbach's plexus)^[5] (Figure 2A). Most EGC are found within the ganglia, and are also present in the interconnecting nerve strands of the ganglionated and in all non-ganglionated plexuses^[6,7].

In the time course, the traditional view of EGC function has changed from simple mechanical support (as their very name, derived from the Greek "glue", implies) to more articulate and complex ones, extremely important for the homeostasis of the gut, including influence on motility and inflammatory processes^[8-10].

In this article we will take into consideration the role of EGC, looking at both experimental animal models and some human diseases for which evidence exists, and in particular their involvement in intestinal motor abnormalities and inflammatory conditions of the gut.

MORPHOLOGY AND IDENTIFICATION OF EGC

Anatomical considerations

These cells are small, with several projecting processes of various length and shapes, which often confer them a star-like appearance^[2,11,12] (Figure 2B). In the ganglia, EGC are very tightly packed around neurons^[2,13] (Figure 3) and extend several flat projections which incompletely insulate enteric neurons from extraganglionic cells^[2,14,15], whereas in the nerve strands glial processes wrap up several axonal bundles^[2,16]. Electron microscopic studies have shown that EGC contain intracellular arrays of 10 nm filaments (mainly constituted by glial fibrillary acidic protein, GFAP^[17-19]) crisscrossing their bodies, forming axial bundles and anchoring the cells to the ganglionic surfaces^[2] (Figure 4).

Moreover, some studies have suggested that EGC in the various plexuses layers of the ENS may be constituted by

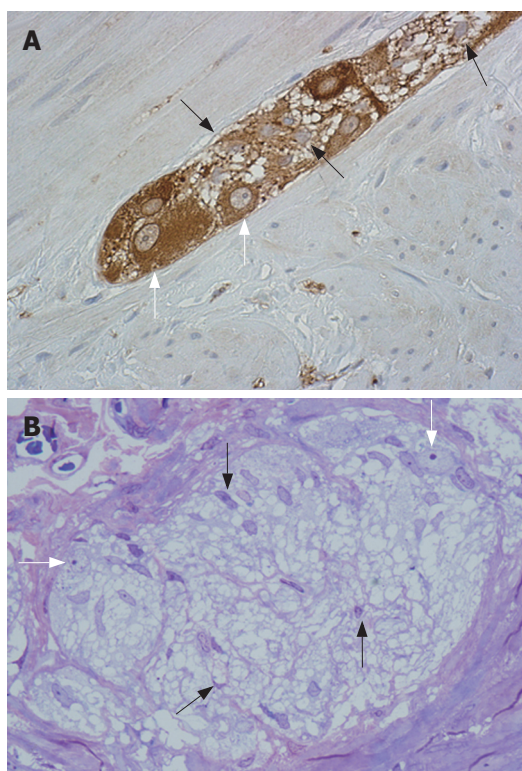


Figure 1 A: Human colonic myenteric plexus showing that neurons (white arrows) are less numerous with respect to EGC (black arrows) (NSE immunostaining, x 40); B: Semithin section of human colonic submucosal plexus, showing the preponderance of EGC (black arrows) with respect to the enteric neurons (white arrows) (Toluidine blue, x 40).

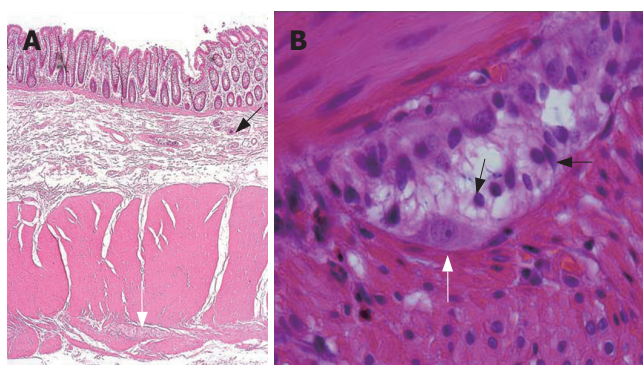


Figure 2 A: Full thickness section of the human colon, showing the submucosal (black arrow) and the myenteric plexus (white arrow) (HE, x10); B: Human myenteric ganglion, showing numerous EGC (black arrows) and an enteric neuron (white arrow) (HE, x 100).

functionally heterogeneous populations^[9,20-22].

Histological and immunohistochemical considerations

The EGC were first described in 1899 with methylene blue staining on full thickness preparations^[23]; today, immunohistochemical methods are most frequently employed for their identification. Mature EGC strongly express vimentin^[24] (also expressed in myofibroblasts)^[25] and GFAP, considered a specific gut glial marker^[17] even though its cellular functions are still obscure^[26]. Another frequently used EGC marker is the S100 protein, which is thought to yield the best results in identifying these cells^[27].

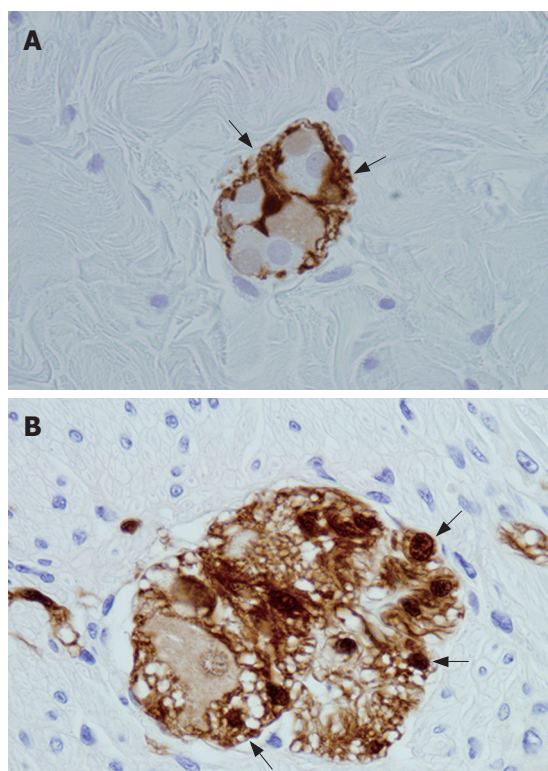


Figure 3 EGC (arrows) tightly packed around enteric neurons in a submucosal (A) and a myenteric ganglion (B) (S100 immunostaining, x 40).

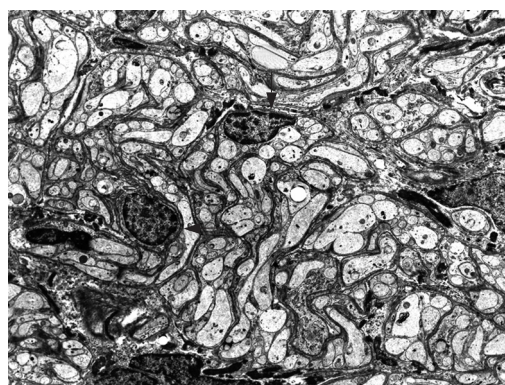


Figure 4 Electron microscopic image of glial cells (arrows) in a human colonic myenteric ganglion (x 1900).

This protein regulates cytoskeletal structure and function and calcium homeostasis in the cytoplasm of glial cells^[28]; in the ENS S100 is thought to be exclusively localized in these cells^[29]. Other putative antibodies for EGC, such as glutamine synthetase (GS)^[30] and the glial cell surface antigen Ran-2^[31] have not been widely employed.

It is also important to underline the fact that EGC, being of neuroectodermal origin^[32], are not related to microglia in the central nervous system, which has a monocyte-macrophage lineage^[33]. Instead, EGC share more similarities with astrocytes, the predominant glial cells of the central nervous system, that regulate synaptic transmission and neurovascular coupling^[34], in addition to be of paramount importance for the formation and function of the blood-neural barrier^[35]. However, it must

be stressed that although EGC display some similarities with astrocytes, there are differences between these two cell populations, such as the dependence on neuregulins in EGC^[9,36] and the functional properties^[9,25]. Finally, the EGC are structurally and functionally different from the Schwann cells of the peripheral nerves (including those within the intestinal wall)^[37,38]; Schwann cells of intramural nerves are S-100-positive but GFAP-negative^[39].

FUNCTIONS OF EGC

Homeostatic function

Experimental evidence suggests that EGC are essential for maintaining the homeostasis of enteric neurons. Studies in animal models demonstrated that the loss of enteric glia causes neuronal degeneration^[40,41] and/or alterations of the neurochemical coding of enteric neurons^[42]. It is thought that the structural and functional integrity of enteric neurons may be due to the glial synthesis of some still unknown trophic factor^[43]. Some studies, for instance, have shown that mature EGC produce glial-derived neurotrophic factor and neurotrophin-3^[44-46], even though no neuronal populations depending on these substances have so far been described in the ENS of mammals. Recent investigations have shown that EGC may have a dipeptide transport function, contributing to the clearance of neuropeptides in the ENS^[47].

ENS mechanical supporting functions

EGC are anchored to the surface of enteric ganglia and nerve strands by means of GFAP bundles^[48], and respond to mechanical stimulation increasing the expression of the immediate-early *c-fos* gene^[49], raising intracellular Ca^{2+} levels and spreading intercellular Ca^{2+} waves^[50]. Thus, it is thought that these cells support and stabilize the ENS through continuous adaptations to the structural and metabolic impairments of the gut wall^[9]. Moreover, EGC express voltage-activated inward and outward K^{+} -channels^[51], suggesting a possible role in preventing extracellular accumulation of K^{+} , which can impair synaptic transmission and ion channel kinetics in the ENS.

Neurotransmitter function

EGC might also be involved in the enteric neurotransmission. In fact, due to the exclusive expression of GS by these cells^[24,30], and the presence of glutamate immunoreactivity in human EGC^[52], enteric glia could have a role in glutamatergic signaling^[9] and represents a source of glutamine for neuronal glutamate and gamma-aminobutyric acid (GABA) resynthesis^[53]. This is further supported by the demonstration that immunoreactivity to the high-affinity GABA transporter GAT2 mostly occurs in EGC^[54], suggesting that the latter might rapidly remove GABA from the extracellular space. Moreover, since EGC but not enteric neurons display immunoreactivity to L-arginine (an essential precursor for nitric oxide)^[55,56], a role in nitrergic neurotransmission might also be possible.

Owing to the fact that EGC propagate intercellular Ca^{2+} waves, an orchestrated intestinal glial activity has been postulated^[50] that would act through a functional network^[57,58]. This network is suggested by the demonstration

of cell-to-cell coupling between EGC (probably through gap junctions)^[12,50,51,58] and the expression of the P2Y4 receptor on these cells^[59]. EGC may also transfer information to the neurons by means of nucleotide signaling^[60-62]. Numerous molecules (serotonin^[60], histamine^[60], endothelin^[63], protease-activated receptors^[64]) can also activate EGC, which increase intracellular Ca^{2+} concentrations^[65] or express the *c-fos* gene, a marker of early cell activation. It has also been shown that EGC express purinoreceptors^[60,66] and that multiple lipid-activated signalling mechanisms exist in these cells^[67,68].

EGC AND GASTROINTESTINAL INFLAMMATION/MOTILITY

EGC and intestinal inflammation

Experimental animal studies have demonstrated that EGC may have a role in intestinal inflammatory processes^[9], and that initiation and/or progression of inflammatory bowel disease (especially Crohn's disease) might be ascribed to an immune-mediated damage to enteric glia^[69]. The fact that EGC functionally interact with lymphocytes^[70-73], respond actively to inflammation, and become activated as antigen-presenting cells^[74] attracting immune cells to the ENS^[9,75], suggests that this cell population is likely involved in inflammatory processes of the gut. Moreover, the immune cells are usually nearby, since the intestine physiologically contains such a cell population that provides a series of pattern recognition receptors interacting with bacterial molecular patterns, and helps to modulate intestinal innate immunity and an appropriate adaptive immune response^[76-78].

Thus, it is not difficult to imagine these cells as active participants in the pathogenesis of the so-called "functional" gastrointestinal disorders. These are usually thought to occur in the absence of anatomical or biochemical abnormalities^[79]. However, this definition now seems outdated, because structural and molecular abnormalities have begun to be recognized in subsets of patients^[80]. For instance, studies in patients with irritable bowel syndrome disclosed the presence of inflammatory infiltrates closely associated with the enteric plexuses and mucosal activation of the immune system^[81,82], and some patients with intestinal dysmotility and megacolon have a lymphoplasmacellular infiltrate within the myenteric plexus that likely accounts for their symptoms^[83].

Evidence for involvement of EGC in abnormal gastrointestinal motility

The role of EGC has been investigated in only a few human diseases, even though there is still no pathological condition entirely ascribable to EGC dysfunction. For instance, patients with colonic diverticular disease have a significant decrease of EGC and of interstitial cells of Cajal (ICC) in the enteric plexuses^[84]. Owing to the fact that in colonic diverticulosis the smooth muscle hypertrophy acts as a partially obstructive mechanism, the EGC population loss might be partly due to this mechanism, similar to that documented for ICC in analogous experimental animal models^[85].

The number of EGC, together with that of enteric neurons and ICC, is also considerably decreased in patients

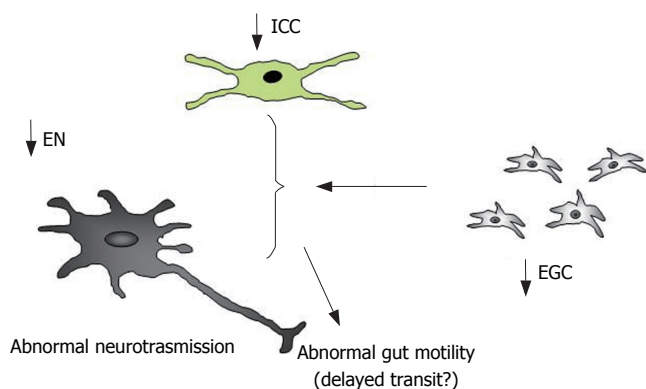


Figure 5 Putative mechanisms linked to the decrease of enteric glial cells (EGC), leading to abnormal gut motility. EN: enteric neurons; ICC: interstitial cells of Cajal.

with severe constipation (slow-transit type) undergoing surgery for intractable symptoms^[86]. Interestingly, the loss of EGC, but not of ICC and enteric neurons, was also documented in the terminal ileum of these patients^[87]; this implies that this cell population may be involved in the small bowel dysmotility repeatedly described in these patients^[88,89]. A significant decrease of EGC, but not of other elements of the ENS, was then described in the myenteric and submucosal plexuses of patients with severe constipation due to obstructed defecation refractory to medical treatment and biofeedback training^[90]. These findings are intriguing, and consistent with the recent hypothesis, based on abnormal colonic manometric findings, that at least one subpopulation of patients with obstructed defecation might result from defective colonic, rather than anorectal, function^[91]. Of practical importance, our results could give an explanation for the lack of response to treatments, especially to biofeedback, in these patients.

More recently, we have reported a significant decrease of EGC in patients with chagasic and idiopathic megacolon compared to controls^[92]; it is worth noting that all the above human pathological conditions in which EGC have been found to be decreased share a common denominator, i.e. constipation.

How can we explain the role of EGC in pathological conditions?

We could hypothesize that the reduced number of EGC, together with the decrease/loss of other cell populations essential for gastrointestinal motility, may play a role in these diseases. For instance, the ICC decrease impairs the pacemaker enteric signals and might add to an abnormal neurotransmission secondary to the decreased number of enteric neurons, and be further worsened by an impairment of EGC. The summation of the loss of the properties of these cell populations might thus lead to dysmotilities of the involved viscera, by means of several mechanisms (Figure 5): impairment of the mechanical properties of the plexuses, decreased gut neurotransmission, and reduced homeostatic support to the enteric neurons, leading to neurodegeneration and/or phenotypic shift, even in the absence of inflammation^[9,93]. Experimental animal models also support this hypothesis, suggesting that EGC play a major role in the modulation of enteric neural circuits that

regulate intestinal motility^[94].

Why EGC, ICC, and enteric neurons are decreased in such patients is still unknown. Evidences in experimental animal models suggest that the number of EGC reduces with aging^[95], but this has not been evaluated in human beings^[96]. Other mechanisms, such as the damaging effect of anthraquinone laxatives on the ENS, have not been confirmed with modern immunohistochemical methods^[97].

CONCLUSION

Probably, the EGC should be looked at differently, since evidence is mounting concerning an ever more active role in the complex organization of the gastrointestinal tract, including enteric neuroplasticity^[98]. The (limited) data so far accumulated suggest that these cells are probably somewhat involved in some motor dysfunction of the gastrointestinal tract, mainly those characterized by constipation. Thus, it is likely that in the future other “functional” disorders of the gut, in addition to the irritable bowel syndrome, may be reclassified. For instance, we have recently proposed consideration of at least some subtypes of constipation such as enteric neuropathies^[99], although seen in the light of the data on EGC we should probably reformulate this definition in terms of neurogliopathies. However, more evidence are needed to establish a more precise role for this fascinating cell population, especially considering new perspectives, such as the possibilities of neural stem cell transplantation^[100-102] for the treatment of disorders of the peripheral and central nervous system. Hopefully, studies on EGC will possibly be useful to establish new therapeutic approaches to some gut disorders.

ACKNOWLEDGMENTS

We are warmly grateful to Professor Giorgio Gabella, Department of Anatomy and Developmental Biology, University College London, London, UK, for kindly providing useful bibliographic references.

COMMENTS

Background

Enteric glial cells (EGC) are to date thought to be more than simple support structures for the enteric nervous system (ENS). Recent developments in their biological properties led to the belief that this cell population may have pathophysiological importance in inflammatory and dysmotility conditions of the gut.

Research frontiers

The EGC have also, in addition to mechanical support function in the ENS, homeostatic functions (are essential for enteric neuronal vitality), neurotransmitter functions, immunological functions (may act as antigen-presenting cells) and appear critically involved in the pathophysiology of inflammatory bowel diseases, especially Crohn's disease.

Innovations and breakthroughs

Recent research in human beings showed that the EGC are likely involved also in the pathophysiological mechanisms of some diseases presenting with abnormal gastrointestinal motility, and especially those characterized by constipation. In fact, significant decreases of EGC have been reported in diverticular diseases, slow transit constipation, some subsets of obstructed defecation, Chagasic and idiopathic megacolon.

Applications

The study of EGC, in addition to that of other components of the ENS (neurons, interstitial cells of Cajal), might result in a better understanding of the pathophysiological grounds of conditions characterized by abnormal gut activity, and perhaps lead to a more targeted therapeutic approach to these disorders.

Peer review

This is an interesting review dealing with the properties of the enteric glial cells, more strictly related to gastrointestinal motor function and the human pathological conditions in which these cells may play a role, suggesting the possibility of enteric neuro-gliopathies.

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S- Editor Zhu LH L- Editor Zhu LH E- Editor Liu Y

EDITORIAL

Endoscopic approach to malignant strictures at the hepatic hilum

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Received: 2007-04-20 Accepted: 2007-05-12

Abstract

Hilar tumors have proven to be a challenge to treat and manage because of their poor sensitivity to conventional therapies and our inability to prevent or to detect early tumor formation. Endoscopic stent drainage has been proposed as an alternative to biliary-enteric bypass surgery and percutaneous drainage to palliate malignant biliary obstruction. Prosthetic palliation of patients with malignant hilar stenoses poses particular difficulties, especially in advanced lesions (type II lesions or higher). The risk of cholangitis after contrast injection into the biliary tree in cases where incomplete drainage is achieved is well known. The success rate of plastic stent insertion is around 80% in patients with proximal tumors. Relief of symptoms can be achieved in nearly all patients successfully stented.

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Key words: Malignant biliary stenoses; Endoscopic retrograde cholangiopancreatography; Endoscopic prostheses

De Palma GD, Masone S, Rega M, Simeoli I, Salvatori F, Siciliano S, Maione F, Girardi V, Celiento M, Persico G. Endoscopic approach to malignant strictures at hepatic hilum. *World J Gastroenterol* 2007; 13(30): 4042-4045

<http://www.wjgnet.com/1007-9327/13/4042.asp>

INTRODUCTION

Extrahepatic malignant stenoses have traditionally been

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separated into three groups, based on anatomical location. Upper third or hilar tumors are those located in the common hepatic duct and/or the right and left hepatic ducts including their confluence. Middle third tumors occur in the region bounded by the upper border of the duodenum and extending to the common bile duct. Lower third or distal bile duct tumors arise between the ampulla of Vater and the upper border of the duodenum.

Malignant biliary obstruction at the liver hilum is caused by a heterogeneous group of tumors that includes primary bile duct cancer (the so-called Klatzkin tumor), cancers that involve the confluence by direct extension (e.g., gallbladder and liver cancer), and metastatic cancer to hilar lymphatic nodes or to the liver or biliary tree.

ANATOMICAL CLASSIFICATION

The extent of duct involvement by perihilar tumours may be classified as suggested by Bismuth and Corlette^[1]: A. type I: tumors below the confluence of the left and right hepatic ducts (ceiling of the biliary confluence is intact; right and left ductal systems communicate); tumors reaching the confluence but not involving the left or right hepatic ducts (ceiling of the confluence is destroyed; bile ducts are separated); C. type III: tumors occluding the common hepatic duct and either the right (IIIa) or left (IIIb) hepatic duct; D. type IV: multicentric tumors or tumors involving the confluence and both hepatic ducts, the right one and the left one (Figure 1).

MANAGEMENT STRATEGIES

Hilar tumors have proven to be a challenge to treat and manage because of their poor sensitivity to conventional therapies and our inability to prevent or to detect early tumor formation. Untreated patients usually die within 6 mo to a year after diagnosis.

The range of therapeutic modalities varies from a curative approach by performing extensive liver resections-in some cases even total hepatectomy and liver transplantation-to a more palliative approach in which a surgical bypass or even percutaneous or endoscopic stent insertion is undertaken, with or without radiotherapy.

All patients should be fully evaluated for resectability before any type of intervention is performed because stent-associated inflammation or infection often makes assessment more difficult.

Patients being evaluated for resectability must at first be physiologically suitable for a potential operative

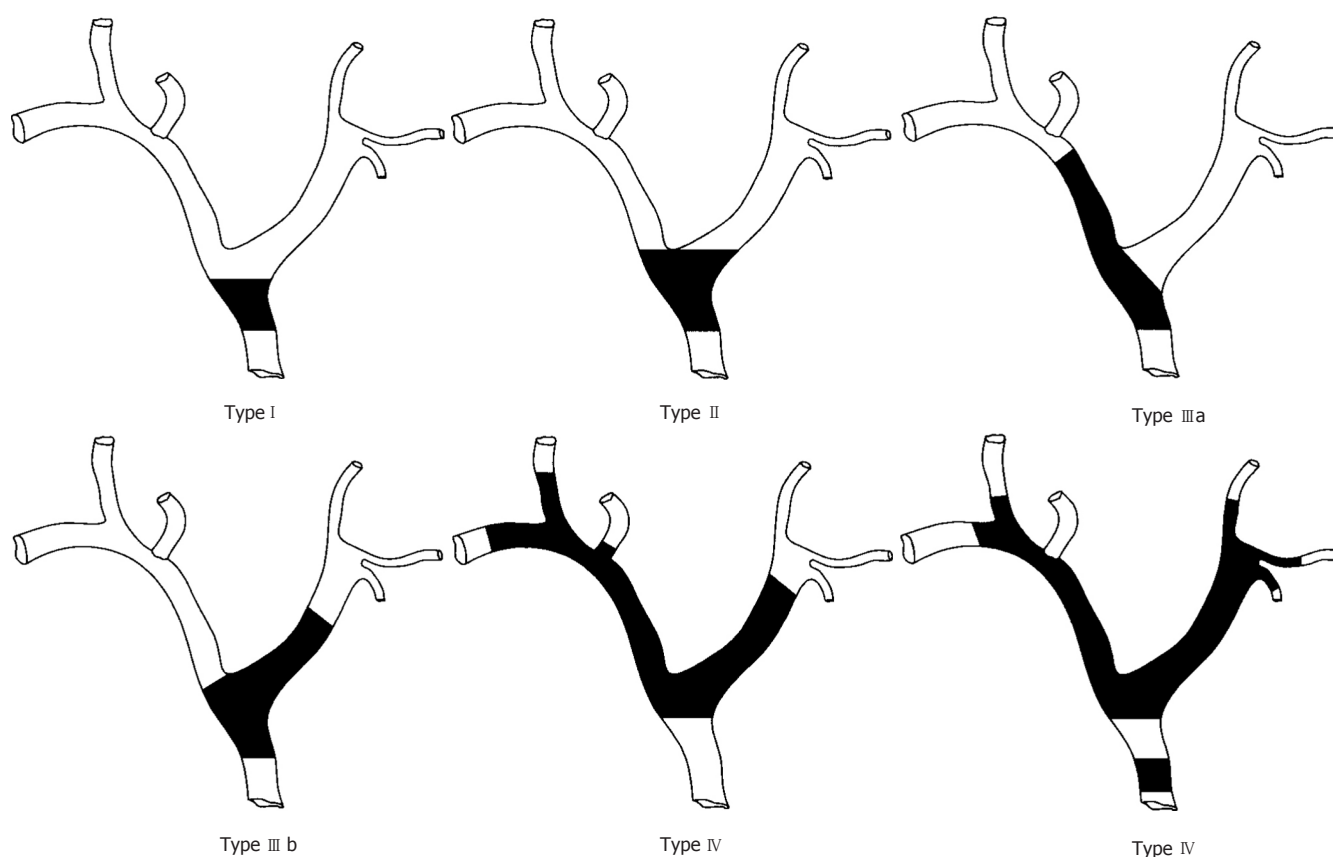


Figure 1 Schematic representation of Bismuth classification of hilar cholangiocarcinoma.

resection that may include a partial hepatectomy. The patient's nutritional status and risk of postoperative liver failure are important factors to consider before proceeding to exploration for resection. A retrospective review of resected hilar cholangiocarcinoma cases demonstrated that a preoperative serum albumin level < 3 g/dL and a total bilirubin level > 10 mg/dL were both associated with poorer survival^[2].

Since the vast majority of extrahepatic strictures, particularly hilar strictures are the result of a cholangiocarcinoma, histological diagnosis is not mandatory before exploration.

The diagnosis of malignant biliary strictures depends on the identification of tumor cells obtained by ultrasound (US) or CT-guided percutaneous fine needle aspiration, bile sampling, endobiliary brushings, or bile duct biopsies. Percutaneous needle biopsies are reliable only if US or CT identifies a malignancy (sensitivity of 50%).

Bile samples, obtained through a percutaneous or endoscopic stent, contain cancerous cells in 30% to 40% of cases of cholangiocarcinoma. The use of brush biopsy and cytologic examination may increase the yield to 40% to 70%. Unfortunately, even percutaneous or endoscopic biopsy not infrequently yields non-diagnostic tissue because of the desmoplastic nature of the lesion.

In the absence of clear evidence of unresectability, all suspected lesions should be considered for resection.

Endoscopic approach

Endoscopic stent drainage has been proposed as an alternative to biliary-enteric bypass surgery and percutaneous drainage to palliate malignant biliary

obstruction. In addition, alternative approaches to biliary stent placement have been compared with particular interest in determining optimal stent material, design, and placement strategies^[3-7].

Prosthetic palliation of patients with malignant hilar stenoses poses particular difficulties, especially in advanced lesions (type II lesions or higher). The risk of cholangitis after contrast injection into the biliary tree in cases where incomplete drainage is achieved is well known. Retention of contrast and subsequent segmental cholangitis is a risk associated with endoscopic attempts to treat advanced hilar lesions and this has prompted some to question the role of endoscopic drainage in this situation^[8].

Some studies suggest that patients undergoing stent placement for malignant low bile duct obstruction had significant improvement in abdominal discomfort, weight loss, or anorexia and sleep patterns, in addition to the expected improvement in pruritus and jaundice^[9]. Similar studies would be needed to confirm that endoscopic stent placement of hilar obstruction is associated with an improved quality of life and can be justified by economic considerations.

Although metabolic and immune parameters appear improved with biliary drainage, there has been no evidence that endoscopic stent placement translates into prolonged survival. The success rate of plastic stent insertion is around 80% in patients with proximal tumors. Relief of symptoms can be achieved in nearly all patients successfully stented.

Technique of stent implantation: The options include draining only the left hepatic system, draining only the

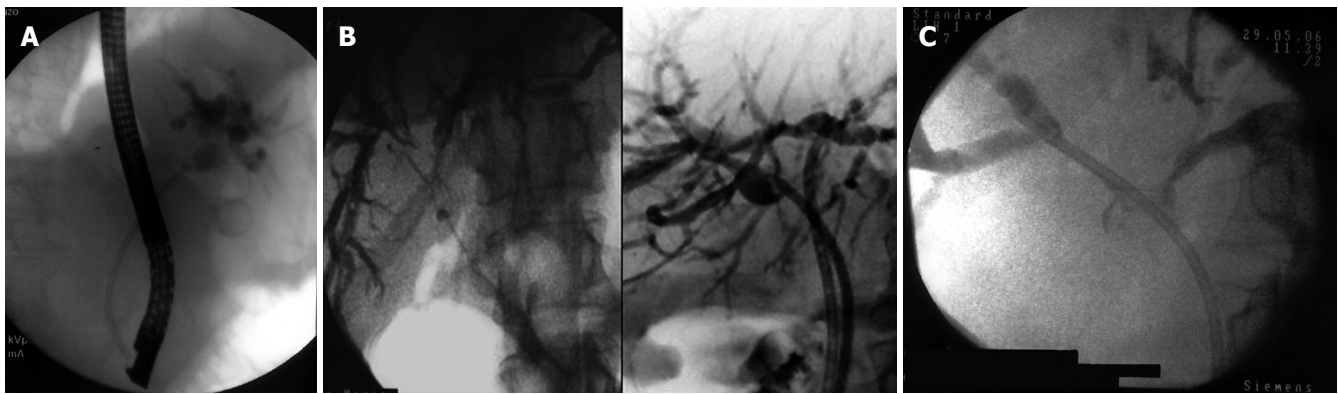


Figure 2 A: Unilateral stent implantation in Bismuth type- I hilar stricture; B: Bilateral stent implantation in Bismuth type III a hilar stricture; C: Unilateral stent implantation in Bismuth type-IV hilar stricture.

right hepatic system, or draining both systems.

The decision whether to place a single biliary stent or multiple stents depends initially on the location of the stricture in the biliary tract.

In patients who have strictures that do not involve the confluence of right and left hepatic ducts (Bismuth type I hilar strictures), jaundice can be palliated completely with a single biliary stent because both the right and left intrahepatic ductal systems are in communication (Figure 2A).

In patients who have more complex strictures (Bismuth type II to IV strictures) the central question is whether adequate palliative relief of obstruction requires the placement of two endoprostheses, one to drain the left system and one to drain the right, or if one prosthesis placed in either system will suffice (Figure 2B and C).

Palliation of jaundice generally requires drainage of 1/4 to 1/3 of a healthy liver, or proportionally more in those with underlying dysfunction. Hence unilateral drainage is usually adequate, and many studies have reported good results using a single stent in about 80% of patients with type II and III tumors. No difference in efficacy has been shown between single stent placement in the left or the right system.

Really, the necessity to ensure the drainage of both systems, including additional endoscopic or percutaneous stent, if necessary, pertains more to the prevention of procedure-induced cholangitis caused by contrast injection in undrained biliary branches than to effective palliation. Generally, if both lobes are imaged with contrast during cholangiography bilateral stenting reduces the potential sequelae of cholangitis in contaminated but undrained areas. If contrast does not contaminate both sides then unilateral stenting should be sufficient^[10-13].

Patients with multiple intrahepatic strictures will probably not benefit from any type of drainage procedure, if several segments (> 1/4) will always remain undrained. In the absence of intractable symptoms, it is probably recommendable that these patients should not undergo further endoscopic measures, as the risk of inducing cholangitis outweighs any benefits that could be possibly realized from the establishment of the endoscopic drainage.

Recent reports describe the utility of magnetic resonance cholangiopancreatography (MRCP) or CT imaging to guide

selection of the target lobe for subsequent endoscopic stenting, often without use of contrast^[12,13].

Selection of the stent (plastic or metal): Theoretically, a metal stent should result in better drainage than a plastic stent in hilar strictures.

The metal stents have two advantages over the plastic stents: it does not occlude side branches because of the multiple meshes; furthermore, because most hilar tumors are firm and scirrhous, tumor ingrowth probably occurs less frequently.

Metallic stents offer longer but still limited stent patency duration of about 4 to 6 mo compared with a patency duration of 2 to 4 mo for plastic stents.

In contrast to plastic stents, metallic stents are not removable after the first few days of deployment, as the stent becomes embedded in the tumor tissue, which may grow into each of the individual mesh opening. Thus, metallic stents should be used in patients with proven unresectable malignancies, because initial insertion of an expandable metal stent makes subsequent surgery more difficult as these stents cannot be readily removed surgically.

The main disadvantage is the cost of the metallic stent (USD 900-1200), and identification of patients who are likely to out-live their first plastic stent, and warrant a metal stent, is a major challenge for the managing clinician. Cost analysis showed that metallic stents were advantageous versus plastic stents in patients surviving more than 6 mo and very costly when patients survived less than three months. Therefore, the use of metal stents should be restricted to those patients with unresectable tumors who will, in all probability, live longer than 3 mo. Unfortunately there is no good way to predict life expectancy at this time. Tumor size (> 3 cm), evidence of diffuse liver metastases, and general condition of the patient could guide the choice of stent.

CONCLUSION

The evaluation of patients with suspected malignancy of the hepatic hilum should include helical or multislice CT of the abdomen. An MRCP should be obtained to assess for resectability. If the disease is resectable and the patient

is fit, surgical resection of the lesion should be performed. Preoperative ERCP should be avoided unless there is cholangitis or significant delay in surgery and the patient is symptomatic. If the lesion is unresectable or the patient is unfit for surgery, then endoscopic palliation of jaundice should be performed by using the MRCP as a guide for unilateral drainage to minimize cholangitis. If cholangitis occurs, ERCP or a percutaneous approach to drain the obstructed lobe of the liver should be performed promptly. The use of metal stents should be restricted to those patients who will, in all probability, live longer than 3 mo.

COMMENTS

Background

Hilar tumors have proven to be a challenge to treat and manage because of their poor sensitivity to conventional therapies and our inability to prevent or to detect early tumor formation. Endoscopic stent drainage has been proposed as an alternative to biliary-enteric bypass surgery and percutaneous drainage to palliate malignant biliary obstruction.

Research frontiers

Alternative approaches to biliary stent placement have been compared with particular interest in determining optimal stent material, design, and placement strategies.

Innovations and breakthroughs

Recent reports describe the utility of MRCP or CT imaging to guide selection of the target lobe for subsequent endoscopic stenting, often without use of contrast.

Applications

Patients with malignant stenoses at hepatic hilum not suitable for surgery.

Terminology

Malignant biliary obstruction at the liver hilum is caused by a heterogeneous group of tumors that include primary bile duct cancer (the so-called Klatskin tumor), cancers that involve the confluence by direct extension (e.g., gallbladder and liver cancer), and metastatic cancer to hilar lymphatic nodes or to the liver or metastases to biliary tree.

Peer review

The authors reviewed about endoscopic approach to hilar malignant strictures, with a focus on endoscopic stent drainage, which has been proposed as an alternative to biliary-enteric bypass surgery and percutaneous drainage to palliate malignant biliary obstruction.

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S- Editor Zhu LH L- Editor Zhu LH E- Editor WangHF

TOPIC HIGHLIGHT

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Hepatorenal syndrome

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Received: 2007-01-11 Accepted: 2007-03-08

Abstract

Hepatorenal syndrome (HRS) is a "functional" and reversible form of renal failure that occurs in patients with advanced chronic liver disease. The distinctive hallmark feature of HRS is the intense renal vasoconstriction caused by interactions between systemic and portal hemodynamics. This results in activation of vasoconstrictors and suppression of vasodilators in the renal circulation. Epidemiology, pathophysiology, as well as current and emerging therapies of HRS are discussed in this review.

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Key words: Acute renal failure; End stage liver disease; Hepatorenal syndrome; Transjugular intrahepatic portosystemic shunts; Dialysis; Liver transplantation

Turban S, Thuluvath PJ, Atta MG. Hepatorenal syndrome. *World J Gastroenterol* 2007; 13(30): 4046-4055

<http://www.wjgnet.com/1007-9327/13/4046.asp>

INTRODUCTION

The association between liver disease and renal dysfunction was reported more than a century ago when patients with chronic liver disease and normal renal histology were found to develop oliguric renal failure (Flint A, *Am J Med Sci* 1863). This led to proposed links between renal dysfunction and the derangement in systemic circulation secondary to the liver failure^[1].

Renal failure in patients with liver disease may be caused by several factors, including shock, sepsis, nephrotoxic

medications, intrinsic renal diseases, or volume depletion secondary to diuresis or large-volume paracentesis. However, renal failure may also occur in patients with liver disease in the absence of the above factors and in the absence of major renal histological changes. This is referred to as hepatorenal syndrome (HRS). HRS is considered a "functional" and reversible form of renal failure^[2-6]. The International Ascites Club defined HRS as: "a syndrome that occurs in patients with advanced chronic liver disease and advanced hepatic failure and portal hypertension characterized by impaired renal function and marked abnormalities in the arterial circulation and activity of the endogenous vasoactive systems. In the kidney, there is marked renal vasoconstriction that results in low glomerular filtration rate (GFR). In the extrarenal circulation, there is a predominance of arterial vasodilation, that results in reduction of total systemic vascular resistance and arterial hypotension". The incidence of HRS in patients with chronic liver disease is not well studied. In one study of 234 non-azotemic patients with liver disease who had ascites and cirrhosis, 18% developed HRS at 1 year, and 39% by 5 years^[7]. Although HRS usually occurs in patients with advanced cirrhosis, it has also been described in patients without ascites in the setting of acute fulminant hepatic failure^[8].

PATHOPHYSIOLOGY

Approximately 80% of hospitalized patients with cirrhosis and ascites have decreased renal perfusion due to moderate vasoconstriction in the renal circulation, which predisposes them to develop HRS^[7-9]. In 10%-17% of these patients, renal vasoconstriction becomes intense enough to cause significant renal hypoperfusion, resulting in HRS^[7,10]. This intense renal vasoconstriction is the distinctive hallmark feature of HRS^[11,12]. The mechanisms of renal vasoconstriction are complex and multifactorial, and are incompletely understood. There appear to be interactions between changes in systemic hemodynamics, portal hypertension, activation of vasoconstrictors, and suppression of vasodilators in the renal circulation^[13,14]. In contrast, significant vasodilation occurs in the splanchnic arterial bed secondary to increased production of local vasodilators, predominantly nitric oxide^[15]. Other vasodilators hypothesized to play a role in splanchnic arterial vasodilation include prostacyclin, prostaglandin E2, atrial natriuretic peptide, kallikreins, and kinins^[10,16,17]. This splanchnic vasodilation is believed to lead to compensatory responses by activating vasoconstrictors including the renin-

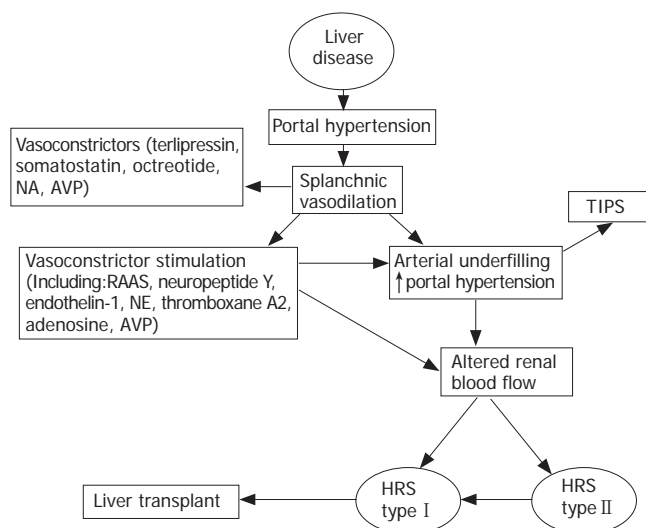


Figure 1 Pathophysiology of HRS and potential therapeutic interventions. NA: noradrenalin; AVP: arginine vasopressin; RAAS: renin-angiotensin system; NE: norepinephrine; TIPS: transjugular intrahepatic portosystemic shunt.

angiotensin-aldosterone system (RAAS), neuropeptide Y, endothelin-1, norepinephrine, thromboxane A₂, adenosine, and antinatriuretic agents such as arginine vasopressin (AVP). This leads to retention of sodium and water in addition to renal vasoconstriction^[15,18,19]. Other factors such as the absence of or decrease in glomerulopressin or other liver-borne diuretic factors (factors that are released by the liver and target the kidney) could also contribute to renal failure^[20]. In recent years, the potential role of cirrhotic cardiomyopathy has been postulated in the pathogenesis of HRS. Ruiz-del-Arbol *et al* have demonstrated that HRS is due to decreased cardiac output in the setting of a severe arterial vasodilation^[21]. Similar circulatory events were also shown in cirrhotic patients who developed spontaneous bacterial peritonitis^[22].

In the early stages of cirrhosis, the activation of local vasodilators may overcome the renal vascular effects of systemic vasoconstrictors, maintaining adequate renal perfusion^[23]. As liver disease progresses, the renal vasodilators are no longer able to antagonize the circulating vasoconstrictors, and this results in severe renal vasoconstriction and impaired renal blood flow. In addition, the hypoperfusion itself may lead to further intrarenal vasoconstriction. Figure 1 summarizes the complex pathways involved in the development of HRS and potential therapeutic interventions.

CLASSIFICATION OF HRS

There are two types of HRS (Table 1). Type 1 is characterized by a rapid elevation in blood urea nitrogen (BUN) and creatinine, often defined as a 100% increase in serum creatinine, reaching a level higher than 2.5 mg/dL in less than two weeks^[2]. The mortality of patients with type 1 HRS has been reported to be 80% at two weeks^[7]. Type 2 HRS, on the other hand, generally follows a slower course and has a better prognosis. It is usually characterized by recurrent, diuretic-resistant ascites^[10,14], and is thought to be due to significant activation of anti-natriuretic systems^[2].

Table 1 Clinical types of HRS

Type 1
(1) 100% increase in serum creatinine to a level higher than 2.5 mg/dL or a 50% reduction of the initial 24-h creatinine clearance to a level lower than 20 mL/min in less than 2 wk
(2) Very poor short-term outcome
Type 2
(1) Serum creatinine > 1.5 mg/dL, without meeting the criteria for type 1 HRS
(2) Refractory ascites is usually present
(3) Prognosis is not as poor as with type 1

PRECIPITATING FACTORS

HRS may develop spontaneously without known precipitating factors, but there are known triggers^[14,24]. Spontaneous bacterial peritonitis (SBP) has been associated with type 1 HRS in approximately 20% of cases^[21,25], even with treatment and resolution of the infection. These patients have a very poor outcome. HRS may also occur after therapeutic paracentesis without plasma expansion^[26,27]. Gastrointestinal bleeding has also been identified as a precipitant of HRS, but this usually occurs in patients with hypovolemic shock. In this setting, acute renal tubular ischemic injury or necrosis is more likely to be the cause of acute renal failure than HRS^[28]. There is no clear evidence to support diuretic-induced volume depletion as a precipitating factor of HRS^[14]. Other factors that have been associated with an increased risk of developing HRS in patients with ascites and cirrhosis include severe urinary sodium retention, spontaneous dilutional hyponatremia, and a mean arterial blood pressure less than 80 mmHg. There is not a direct linear association between the severity of liver failure and the incidence of HRS, but HRS is usually seen in patients with advanced liver disease and portal hypertension^[14,23].

DIAGNOSIS OF HRS

There are no specific clinical or laboratory findings for the diagnosis of HRS. The diagnosis is established based on predefined criteria in the appropriate clinical setting (Table 2). Patients with advanced liver disease may develop renal failure from a number of causes other than HRS, and these causes must be excluded before making a diagnosis of HRS. Common causes of renal failure in patients with cirrhosis include volume depletion (which could be secondary to over-diuresis, diarrhea, or poor fluid intake), nephrotoxic medications (commonly non-steroidal anti-inflammatory agents and aminoglycosides), allergic interstitial nephritis, acute tubular necrosis (from various factors including shock), contrast nephropathy, and intrinsic renal diseases such as glomerulonephritis. A renal biopsy may rarely be necessary if the diagnosis is unclear, mainly to exclude other treatable renal diseases. It is also important to note that there are significant limitations in using serum creatinine as a marker of renal function in patients with liver disease. Patients with advanced liver disease usually have reduced muscle mass and hence low endogenous production of creatinine. When creatinine clearances in cirrhotic patients were compared with inulin clearances, the

glomerular filtration rates were significantly overestimated^[29]. Alternative diagnostic approaches have been applied in order to overcome the limitation of serum creatinine values in this population. Platt *et al*^[9] examined the utility of Doppler ultrasonography to assess the resistive indices of the renal vasculature. In their study of 180 patients with liver disease without azotemia, 42% of the patients were found to have an increase in renal vascular resistive indices. Of those patients, 55% subsequently developed renal failure as compared to 6% of those with normal resistive indices. The sensitivity and specificity of the resistive index in detecting renal failure were estimated at 71% and 80% respectively in a group of cirrhotic patients^[30]. However, this technique is operator-dependent and is still under investigation, and therefore is not currently recommended as a standard method to diagnose HRS.

PROGNOSIS

The prognosis of HRS is extremely poor. The median survival time of patients with type 1 HRS is less than 2 wk, with less than 10% surviving their hospital stay^[7]. The survival time of patients with type 2 HRS, although still short, is significantly longer, with a median survival time of approximately 6 mo^[14].

MANAGEMENT

Prevention of HRS

Prevention of HRS is potentially possible in some high-risk patients. In patients with SBP, administering intravenous albumin (1.5 g/kg upon diagnosis, and then 1 g/kg after 48 h) in addition to antibiotics has been shown to decrease the incidence of HRS and to decrease hospital mortality as compared with treatment with antibiotics alone^[26]. The authors of that study postulated that the administration of albumin prevented circulatory dysfunction by maintaining effective arterial blood volume and therefore prevented vasoconstrictor activation. However, albumin is expensive, and more studies are needed to determine if lower doses of albumin or less expensive artificial plasma expanders are as effective. In one study, administration of pentoxifylline (400 mg orally three times a day) to patients with severe acute alcoholic hepatitis decreased the incidence of HRS as well as the short-term mortality rate compared to placebo^[31]. This benefit may be related to the inhibition of tumor necrosis factor production. Although both of the above studies support the idea of preventing renal failure in the setting of liver failure, there are no data evaluating the long-term survival benefit in this population. Moreover, there have been no further confirmatory studies.

General management measures

In patients with type 1 HRS, diagnostic paracentesis is generally recommended to evaluate for SBP. In addition, diuretics should be discontinued as they may potentially worsen renal function. In the absence of contraindications, patients with type 1 HRS should also be evaluated for expedited liver transplantation.

Table 2 Diagnostic criteria of hepatorenal syndrome¹

Major criteria (all must be present for the diagnosis of HRS)
(1) Advanced hepatic failure (acute or chronic liver disease) and portal hypertension
(2) Low GFR defined as serum creatinine > 1.5 mg/dL or creatinine clearance < 40 mL/min
(3) Absence of shock, significant volume losses, ongoing infection, or treatment with nephrotoxic medications
(4) Absence of a sustained improvement in renal function after cessation of diuretics and expansion of plasma volume with 1.5 L of isotonic fluids
(5) Urine protein excretion < 500 mg/dL with no ultrasonographic evidence of obstruction or parenchymal renal disease
Additional criteria (not necessary for the diagnosis, but provide supportive evidence)
(1) Urine volume < 500 mL/d
(2) Urine sodium < 10 mEq/L
(3) Urine osmolality greater than plasma osmolality
(4) Urine red blood cells < 50 per high-power field
(5) Serum sodium concentration < 130 mEq/L

¹Adapted from Arroyo *et al*^[2].

Pharmacological therapy

Several systemic vasoconstrictors have been utilized in the treatment of type 1 HRS as summarized in Table 3. Renal vasodilators such as dopamine and prostaglandin analogues are no longer recommended due to their side effect profile and the lack of clinical evidence to support their use. Other potential forms of therapy that have not been extensively tested include endothelin blockers^[32] and N-acetylcysteine^[33].

The rationale behind the use of vasoconstrictors along with plasma expansion is that they will counteract the splanchnic arterial vasodilation, which is hypothesized to be the initial event in the pathogenesis of HRS. Unopposed splanchnic arterial vasodilation may cause a decrease in effective arterial volume which in turn triggers the activation of vasoconstrictors^[23,34]. Vasoconstrictors that have been widely used for type 1 HRS include vasopressin analogues (ornipressin and terlipressin), a somatostatin analogue (octreotide), and alpha-adrenergic analogues (midodrine and noradrenalin). In most studies, albumin was administered concurrently.

The vasopressin analogues are effective in causing marked splanchnic vasoconstriction. Ornipressin, although effective in treating HRS, may cause significant ischemic side effects and is not currently recommended for the management of HRS^[35]. Studies using terlipressin, the long-acting analogue of vasopressin, have shown significant improvement in renal function in approximately 60%-75% of patients, with a lower than 5% incidence of ischemic side effects^[36-43]. In these studies, patients with Child-Pugh scores less than or equal to 13 and/or those who received albumin infusions had a more favorable outcome. However, it is important to note that GFR was not normalized in most patients who responded^[37,39]. Approximately 15% of patients had recurrence of HRS once treatment was discontinued. Small, short-term, non-randomized studies suggest that treatment with terlipressin may also improve renal function in patients with type 2 HRS^[34]. Terlipressin is not currently licensed

Table 3 Non-invasive therapies

Author and Year	n (# Type 1, # Type 2)	Study design	Intervention	Outcome measures	Mean baseline SCr	Mean follow-up SCr	Other results	Comments
Moreau <i>et al</i> 2002 ^[38]	99 (99/0)	Multicenter, retrospective	Terlipressin (75% received albumin)	Reduction of SCr to < 130 μ mol/L or a decrease of at least 20% at end of treatment)	272 \pm 114 μ mol/L	Responders: 138 \pm 59 μ mol/L Nonresponders: 382 \pm 210 μ mol/L	Renal function improved in 58% of patients.	Twenty-three patients had adverse events that may have been terlipressin-related. Three patients required RRT 40% survival at 1 mo.
Kiser <i>et al</i> 2005 ^[44]	43 (32/11)	Observational (retrospective cohort)	Vasopressin (AVP) <i>vs</i> octreotide <i>vs</i> combination	Clinical response; SCr 1.5 mg/dL or less	3.9 \pm 3.3 mg/dL	Responders: SCr decreased by 62% \pm 9% Nonresponders: SCr increased by 46% \pm 119%	42% complete response with AVP <i>vs</i> 38% with AVP and octreotide <i>vs</i> 0% with octreotide alone.	No adverse effects related to AVP. RRT rates: 50% in AVP group, 58% in combination group, and 31% in octreotide alone group.
Solanki <i>et al</i> 2003 ^[43]	24 (24/0)	Randomized placebo-controlled single-blind	Terlipressin <i>vs</i> placebo (all patients received albumin) for 4-15 d	Reversal of HRS and survival at 15 d	Terlipressin: 2.9 \pm 0.1 mg/dL Placebo: 2.2 \pm 0.2 mg/dL	Terlipressin: 1.2 \pm 0.2 mg/dL at d 15 Placebo: no survival at d 15 (SCr 3.9 \pm 0.2 mg/dL on d 8)	In terlipressin group, 5 of 12 patients survived. None survived by d 15 in placebo group.	
Ortega <i>et al</i> 2002 ^[39]	21 (16/5)	Prospective, nonrandomized	Terlipressin with albumin <i>vs</i> without albumin for 4-14 d	SCr 1.5 mg/dL or lower	Terlipressin with albumin: 3.6 \pm 1.5 mg/dL Terlipressin without albumin: 3.4 \pm 0.3 mg/dL	Terlipressin with albumin: 1.5 \pm 0.2 mg/dL Terlipressin without albumin: 3.4 \pm 0.7 mg/dL	10 of 13 patients who received terlipressin and albumin responded. Of 8 patients who received terlipressin alone, 2 responded.	One patient had ischemic side effects (finger ischemia). At 1 mo, there was a 5% recurrence of HRS after complete response.
Pomier-Layrargues <i>et al</i> 2003 ^[61]	19 (NS)	Randomized, double-blind, placebo-controlled, crossover	Placebo, then octreotide (Group 1) <i>vs</i> octreotide, then placebo (Group 2) (all patients received albumin)	20% decrease in SCr after 4 d	Group 1: 215 \pm 32 μ mol/L Group 2: 208 \pm 16 μ mol/L	Group 1: 222 \pm 41 μ mol/L after placebo; 270 \pm 54 μ mol/L after octreotide Group 2: 194 \pm 34 μ mol/L after octreotide; 204 \pm 47 μ mol/L after placebo	Treatment with octreotide was not effective.	The study included types 1 and 2 HRS patients (numbers in each group not specified). No side effects reported.
Colle <i>et al</i> 2002 ^[42]	18 (18/0)	Chart review (retrospective analysis)	Terlipressin (some patients received albumin)	Decrease in SCr to < 130 μ mol/L or decrease of at least 20% leading to a stable value; evaluation of predictive factors	Patients with improved SCr: 276 \pm 47 μ mol/L ¹ Patients without improved SCr: 295 \pm 89 ¹ μ mol/L	Patients with improved SCr: 130 \pm 13 μ mol/L Patients with improved SCr: 411 \pm 89 μ mol/L	11 patients had improved renal function	Some of these patients were included in the Moreau study. Patients with improved renal function had less severe cirrhosis than patients without. Patients without a precipitating factor for HRS or who responded to terlipressin were more likely to survive.
Halimi <i>et al</i> 2002 ^[41]	18 (16/2)	Multicenter pilot (retrospective)	Terlipressin for 2-16 d	> 30% decrease in baseline SCr	298 \pm 124 μ mol/L	145 \pm 85 μ mol/L	13 of 18 had improved renal function; 8 had a normal SCr at d 5	Three patients had ischemic side effects. One had severe bronchospasms after terlipressin administration, and subsequently died.

Guevara <i>et al</i> 1998 ^[49]	16 (Type NS)	Open pilot study	Ornipressin and albumin for 3 vs 15 d	Efficacy	3-d arm: 2.9 ± 0.5 mg/dL 15-d arm: 3.0 ± 0.5 mg/dL	3-d arm: 2.2 ± 0.4 mg/dL 15-d arm: 0.7 ± 0.1 mg/dL	75% of patients had improved renal function.	Treatment was stopped in 4 patients on the 15-d protocol because of ischemic complications.
Angeli <i>et al</i> 1999 ^[62]	13 (13/0)	Nonrandomized	Dopamine and albumin (Group A) vs midodrine, octreotide, and IV albumin (Group B)	Efficacy	Group A: 3.6 ± 0.6 mg/dL Group B: 5.0 ± 0.9 mg/dL	Group A: 5.1 ± 1.5 mg/dL at 15 d (only 1 patient survived to d 20) Group B: 3.3 ± 0.7 mg/dL at 20 d	All Group B patients had improved GFR. 7 of 8 patients in Group A had worsening renal function and died.	No significant side effects.
Holt <i>et al</i> 1999 ^[33]	12 (NS)	Open label	N-acetylcysteine for 5 d	Efficacy	222 ± 27 μmol/L	169 ± 7 μmol/L		67% survival at 1 mo, and 58% at 3 mo (2 patients received liver transplants).
Mulkay <i>et al</i> 2001 ^[40]	12 (12/0)	Pilot	Terlipressin for 1 wk to 2 mo	Safety and efficacy	3.4 mg/dL	1.8 mg/dL		Three patients received liver transplants, and had near-normal renal function. The other 9 died during follow-up. No ischemic complications.
Duvoux <i>et al</i> 2002 ^[48]	12 (12/0)	Pilot	Noradrenalin (NA), albumin, and furosemide for at least 5 d	Safety and efficacy	2.6 ± 1.1 mg/dL pre-furosemide/albumin; 3.9 ± 1.8 mg/dL after infusion (pre-NA)	1.6 ± 0.8 mg/dL	Reversal of HRS in 10 of 12 patients	Two patients had previously received terlipressin (underwent 48-h washout before starting NA). Transient myocardial ischemia was observed in 1 patient. No side effects reported.
Hadengue <i>et al</i> 1998 ^[63]	9 (9/0)	Double-blind, short-term, controlled crossover study	Terlipressin and placebo for 2 d in randomized order	Efficacy	Baseline CrCl: 15 ± 2 mL/min	CrCl after terlipressin (includes both groups): 27 ± 4 mL/min CrCl after placebo (includes both groups): 16 ± 3 mL/min		No side effects reported.
Uriz <i>et al</i> 2000 ^[37]	9 (6/3)	Pilot	Terlipressin with albumin for 5-15 d	Reduction of serum creatinine to < 1.5 mg/dL	3.9 ± 0.7 mg/dL	1.5 ± 0.2 mg/dL	Reversal of HRS in 7 of 9 patients.	One patient did not complete the study due to pancreatitis. No ischemic complications.
Angeli <i>et al</i> 1998 ^[45]	8 (0/8) + 17 cirrhotic patients without HRS	Open label	Midodrine (one dose)	Renal function and renal hemodynamics (acute effects)	GFR: 39.0 ± 6.4 mL/min	GFR: 45.1 ± 7.6 mL/min	No significant acute effect on renal hemodynamics or renal function.	This study looked at the acute effect of one dose of midodrine. The results include cirrhotic patients without HRS.
Gulberg <i>et al</i> 1999 ^[64]	7 (7/0)	Nonrandomized	Orinpressin, dopamine, and albumin for 5-27 d	2× increase in Crcl (to > 40 mL/min)	Treatment success group: 4.6 ± 0.9 mg/dL, and improved to 1.3 ± 0.2 mg/dL	Treatment success group: 1.3 ± 0.2 mg/dL	HRS was reversed in 4 of 7 patients	Two responders had a relapse. One of them responded to retreatment, but treatment was stopped in the other because of a ventricular tachyarrhythmia. Treatment was stopped in another patient because of intestinal ischemia.

Kaffy <i>et al</i> 1999 ^[65]	5 (NS)	Pilot	Octreotide for 5 d	Efficacy	194 μ mol/L in 4 patients	96 μ mol/L in 4 patients	Improvement of SCr in 4 of 5 patients,	but 4 of 5 patients eventually died. HRS rapidly recurred when octreotide was stopped, and did not respond to further octreotide infusion.
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SCr: Serum Creatinine; RRT: Renal replacement therapy; NS: Not Specified; CrCl: Creatinine Clearance. Cr conversion from μ mol/L to mg/dL: divide by 88.4.

Table 4 Invasive therapies

Author and Year	N (# Type 1, # Type 2)	Study design	Intervention	Outcome measures	Mean baseline SCr	Mean follow-up SCr	Other results	Comments
Brensing <i>et al</i> 2000 ^[50]	31 (14/17); an additional 10 were too sick to receive TIPS	Phase II	TIPS	Safety and survival	(Of the 31 patients who received TIPS) 2.3 \pm 1.7 mg/dL	Wk 4: 1.5 \pm 1.2 mg/dL	Renal function improved within 2 wk after TIPS and subsequently stabilized.	Three-month survival rate was 81% (10% of non-shunted patients survived, but they were felt to be too sick to receive TIPS). There was 1 TIPS-related death.
Wong <i>et al</i> 2004 ^[46]	14 (14/0)	Prospective	Midodrine, octreotide, albumin, and TIPS	Efficacy (serum creatinine < 135 μ mol/L for at least 3 d)	Responders: 233 \pm 29 μ mol/L Nonresponders: 345 \pm 83 μ mol/L	Responders: 112 \pm 8 μ mol/L after medical therapy Nonresponders: 476 \pm 139 μ mol/L after medical therapy.	Renal function improved in 10 of 14 patients (71%) with medical therapy. Five responders received TIPS; their renal function continued to improve. Mean GFR was 96 \pm 20 mL/min by 12 mo post-TIPS.	TIPS was performed in responders who were stable. Two of the five responders who did not receive TIPS underwent liver transplantation, and their SCr remained normal at the time of liver transplantation.
Alessandria <i>et al</i> 2002 ^[36]	16 (0/11, and an additional 5 with "organic renal disease")	Prospective, nonrandomized	Terlipressin for 7 d (and TIPS in stable patients)	Efficacy	2.4 \pm 0.9 mg/dL	After terlipressin therapy: 1.8 \pm 0.8 mg/dL After TIPS: 1.4 \pm 0.3 mg/dL	Terlipressin: 8 of 11 HRS patients had improved renal function (and 7 of the 8 responders had reversal of HRS (SCr < 1.5 mg/dL). Subsequent TIPS: 8 of 9 patients (89%) who underwent TIPS had improved renal function by 1 mo.	Renal function improved significantly after TIPS in all patients who responded to terlipressin. One HRS patient who did not respond to terlipressin underwent TIPS and responded. In the non-HRS group (with "organic renal disease", only one patient had an improved SCr (from 3.7 to 1.8 mg/dL) with terlipressin treatment.
Guevara <i>et al</i> 1998 ^[49]	7 (7/0)	Prospective	TIPS	Efficacy	4.9 \pm 0.8 mg/dL	1 wk after TIPS: 3.7 \pm 1.0 mg/dL 1 mo after TIPS: 1.8 \pm 0.4 mg/dL	Renal function improved in 6 of 7 patients.	Mean survival was 4.7 \pm 2 mo.
Witzke <i>et al</i> 2004 ^[53]	30 (NS)	Prospective	CVVHD (if mechanically ventilated) vs intermittent HD if not ventilated	Survival	N/A	N/A	8 of 15 patients who received HD survived. None of the ventilated patients (received CVVHD) survived.	Note that the sickest patients (on a ventilator) all received CVVHD.

Keller <i>et al</i> 1995 ^[54]	26 (NS); an additional 81 patients had liver disease and renal failure, but were not diagnosed with HRS	Retrospective	HD	Risk factor evaluation and outcomes	N/A	N/A	7 of 16 patients with HRS who received HD survived, while only 1 out of 16 patients with HRS who did not receive HD survived.	
Mitzner <i>et al</i> 2000 ^[55]	13 (Type 1)	Prospective, randomized, controlled	MARS and HDF and standard medical therapy <i>vs</i> HDF and medical therapy	Survival	MARS + HDF: 3.8 ± 1.5 mg/dL HDF alone: 4.4 ± 1.3 mg/dL	MARS + HDF: 2.3 ± 1.5 mg/dL HDF alone: 3.8 ± 0.5 mg/dL	At one week: 62.5% mortality in the treatment group, and 100% mortality in the group who did not receive MARS.	None of these patients underwent liver transplantation or received TIPS or vasopressin analogues during the observation period.
Jalan <i>et al</i> 2003 ^[66]	8 (5/2, and one patient without HRS)	Prospective, nonrandomized	MARS	Safety and efficacy	162 (51–312) μmol/L	108 (34–231) μmol/L	50% survival at 3 mo follow-up	All of the patients had alcoholic hepatitis and were encephalopathic. Renal function improved in all patients. Of the 5 patients with type 1 HRS, 3 remained anuric, but there was normalization of SCr in the other 2 patients. SCr was normalized in both patients with type 2 HRS by the end of treatment.
Mitzner <i>et al</i> 2001 ^[55]	8 (NS)	Uncontrolled	MARS	Multiple organ function changes	380 ± 182 μmol/L	163 ± 119 μmol/L	Improvement in multiple organ functions	

SCr: Serum creatinine; CrCl: Creatinine clearance; TIPS: Transjugular intrahepatic portosystemic shunt; NS: Not Specified; CVVHD: Continuous veno-venous hemodialysis; HD: Hemodialysis; MARS: Molecular adsorbents recirculating system; HDF: Hemodiafiltration. Cr conversion from μmol/L to mg/dL: divide by 88.4.

for use in North America, but a double-blind, randomized, placebo-controlled trial is now being conducted in the USA and Germany in patients with type 1 HRS. Alpha-1 adrenoreceptor agonists and a somatostatin analogue are readily available in North America and have been studied in type 1 HRS. An observational study compared vasopressin infusion with octreotide infusion in patients with HRS, and found a complete response rate of 41% in the patients treated with vasopressin compared with 0% in the patients treated with octreotide^[44]. In type 1 HRS, alpha-1 agonists have only been used in combination with other agents. Few nonrandomized, prospective studies have evaluated treatment with both midodrine and octreotide^[45–47]. The study by Angeli^[45] included only five patients and showed that after 20 d of treatment, all patients had serum creatinine levels below 2 mg/dL. In the study by Wong *et al*^[46], 10 of 14 patients with HRS treated with midodrine, octreotide, and albumin had their serum creatinine stable at less than 1.5 mg/dL for three days. The use of noradrenalin in combination with

intravenous albumin and furosemide was studied in 12 patients^[48]. HRS was reversed in 83% of patients, with an adverse event rate of 17%. These small studies suggest a short-term benefit in improving renal function in HRS patients, although larger, randomized studies are required before recommending the routine use of these agents in clinical practice. Other drugs, such as N-acetylcysteine and misoprostol, have been proposed as therapy for HRS, but have not been well-studied.

Non-pharmacologic therapy

Transjugular intrahepatic portosystemic shunts (TIPS), by reducing portal hypertension, may be useful in treating HRS (Table 4), although no trials have shown a survival advantage^[49–51].

Renal replacement therapy

Patients with HRS who progress to severe renal failure can be initiated on renal replacement therapy (RRT), generally given as continuous hemofiltration. Dialysis

is usually used as a bridge in patients who are awaiting liver transplantation, and is not recommended for patients who are unlikely to recover from liver failure or are unlikely to receive liver transplantation because of other contraindications. Survival on dialysis is generally dependent on the severity of liver disease^[52]. There are only a few small studies evaluating the effects of dialysis in HRS^[53,54]. Keller *et al.*^[54], in a retrospective study, found that 7 of 16 patients with HRS who received RRT survived, while only 1 out of 16 patients with HRS who did not receive RRT survived. In the prospective study by Witzke *et al.*^[53], 30 patients with Child-Pugh C cirrhosis and HRS were treated with continuous veno-venous hemodialysis (CVVHD) if they were on mechanical ventilation, or with intermittent hemodialysis if they were not on mechanical ventilation. No patients on mechanical ventilation survived for more than 30 d, but 8 of 15 patients who were not on mechanical ventilation survived for more than 30 d. The absence of a control group and lack of randomization make it difficult to draw any firm conclusions from this study.

Molecular absorbent recirculating system (MARS) is a form of albumin dialysis, which removes “toxins” such as tumor necrosis factor- α , interleukin-6, and nitric oxide *via* binding to dialysate albumin. A small, randomized trial showed a survival advantage of MARS when compared to standard therapy in HRS patients^[55]. To date, there have been no other published trials comparing MARS to standard RRT.

Transplantation

Liver transplantation (LT) is the only effective and permanent treatment for HRS^[10,14,56,57] that cures both the liver and renal failure. However, the 5-year survival rate in LT recipients with HRS is significantly less than in LT patients without HRS^[56]. Patients who undergo LT may sometimes require postoperative hemodialysis. It is preferable to delay administration of cyclosporine or tacrolimus until renal impairment improves in these patients, as these drugs may further worsen renal function. The issue of whether to transplant a kidney in addition to a liver (LKT, combined liver kidney transplantation) is an important one as well. HRS alone is not considered an indication for a LKT^[58]. A renal biopsy may be helpful in some patients to identify the etiology of the renal failure and to determine the presence and extent of glomerular scarring^[59]. LKT should be reserved for patients with irreversible renal failure, including HRS patients who are on dialysis for more than 8 wk or patients with progressive primary renal disease^[59]. United Network of Organ Sharing (UNOS) data indicate a 5-year survival of LKT recipients of 62% compared with 50% for patients with a serum creatinine > 2.0 mg/dL receiving isolated LT ($P = 0.0001$). One center's results demonstrated a 5-year patient survival of 48% for LKT patients, 67% for HRS patients receiving isolated LT, and 70% for patients with a serum creatinine > 2.0 mg/dL receiving isolated LT (P not significant comparing all groups)^[58]. It is not clear if the advances in management of HRS in recent years have had an impact on post-transplant outcomes. In a case-control study by Restuccia *et al.*, patients with HRS

treated with vasopressors and albumin prior to LT had similar survival outcomes compared to those patients who underwent OLT without HRS^[60]. However, the study had only 9 patients with HRS and as correctly stated by the authors, further confirmation in a larger series of patients is required. Clearly, further prospective studies are needed to guide transplant physicians to determine whether they should transplant the liver and the kidney or the liver alone in patients with liver failure and kidney failure.

CONCLUSION

Renal failure occurs commonly in patients with severe liver disease and its causes are multifactorial. Patients with type 1 HRS generally have a fatal outcome without expedited liver transplantation. Therapy with terlipressin and albumin looks promising, but there is a paucity of data to make firm conclusions. Use of other vasoconstrictors or TIPS remains experimental. The only proven treatment option is expedited liver transplantation. Dialysis is often used as a bridge to liver transplantation, but there are no controlled studies to support renal replacement therapy in type 1 HRS. Further research is necessary to better elucidate the mechanisms of HRS and to identify treatment strategies to reduce morbidity and mortality in patients with liver disease.

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S- Editor Liu Y E- Editor Lu W



TOPIC HIGHLIGHT

Paul Joseph Thuluvath, Professor, Series Editor

Outcomes of patients with cirrhosis undergoing non-hepatic surgery: Risk assessment and management

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Received: 2007-04-10 Accepted: 2007-05-12

Abstract

The reported mortality rates in patients with cirrhosis undergoing various non-transplant surgical procedures range from 8.3% to 25%. This wide range of mortality rates is related to severity of liver disease, type of surgery, demographics of patient population, expertise of the surgical, anesthesia and intensive care unit team and finally, reporting bias. In this article, we will review the pathophysiology, morbidity and mortality associated with non-hepatic surgery in patients with cirrhosis, and then recommend an algorithm for risk assessment and evidence based management strategy to optimize post-surgical outcomes.

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Key words: Pre-operative risk assessment; Risk stratification; Cirrhosis; Model for end-stage liver disease; Non-transplant surgery; Outcomes

Millwala F, Nguyen GC, Thuluvath PJ. Outcomes of patients with cirrhosis undergoing non-hepatic surgery: Risk assessment and management. *World J Gastroenterol* 2007; 13(30): 4056-4063

<http://www.wjgnet.com/1007-9327/13/4056.asp>

INTRODUCTION

The prevalence of chronic liver disease is increasing in the US and the rest of the world because of hepatitis C (HCV), hepatitis B (HBV), alcohol and non-alcoholic fatty liver disease (NAFLD)^[1]. It has been estimated that about 10% of patients with cirrhosis will undergo surgery in the last 2 years of their life^[2]. General anesthesia and surgery

may lead to complications in a significant proportion of patients with well-compensated or occult cirrhosis, and these complications may result in considerable morbidity and mortality. The reported mortality rates in patients with cirrhosis undergoing various surgical procedures range from 8.3% to 25% (Table 1) in comparison to 1.1% in non-cirrhotic patients^[3-6]. This wide range of mortality rates is related to severity of liver disease, type of surgery, demographics of patient population, expertise of the surgical, anesthesia and intensive care unit (ICU) team and finally, reporting bias. It is therefore, important to assess the risk in relation to the type of surgery that is performed; arbitrarily, the type of surgery could be categorized into high, moderate or low risk surgery (Table 2)^[3]. In this article, we will review the morbidity and mortality associated with non-hepatic surgery in patients with cirrhosis, and then recommend a risk assessment and evidence based management strategy to optimize post-surgical outcomes^[7,8].

MORBIDITY, MORTALITY AND RISK STRATIFICATION

The published evidence for the risk of surgery in patients with cirrhosis is mostly derived from retrospective studies. These have shown that the postoperative mortality and morbidity correlate well with Child-Turcotte-Pugh (CTP) class of cirrhosis (Table 3)^[3,9]. Based on these reports, it has been suggested that elective surgery is tolerated in patients with CTP class A cirrhosis, permissible with preoperative preparation in patients with CTP class B cirrhosis (except those undergoing extensive hepatic resection or cardiac surgery) and contraindicated in patients with CTP class C cirrhosis (Table 4). In recent years, the model for end-stage liver disease (MELD) score has been utilized to prioritize organ allocation in patients awaiting liver transplant. This objective score has been shown to reflect the 90 d mortality in patients with cirrhosis. Unlike the CTP score which has subjective components (severity of hepatic encephalopathy and ascites), the MELD score is considered more objective as it relies only on serum bilirubin, creatinine and international normalized ratio (INR). More recent studies suggest that MELD could also be used to stratify risk in patients undergoing non-transplant surgery, and MELD score < 10, 10-14 and > 14 may correspond to CTP classes A, B and C respectively^[9,10].

Other proposed individual risk factors include the presence of jaundice, prolonged prothrombin time

Table 1 Reported surgical mortality risk in patients with cirrhosis

Surgery in patients with cirrhosis	n	Died (n)	%	Ref No.
Gastrointestinal				
Peptic ulcer surgery-elective	20	2	10	34
Peptic ulcer surgery-bleeding	94	46	49	34
Peptic ulcer surgery-perforation	167	70	42	34
Gastric bypass	125	5	4	38
Biliary tract surgery	212	45	21	34, 39
Small bowel surgeries	9	6	67	34
Colon surgeries	87	36	41	34
Open cholecystectomy	110	8	7.7	40
Laparoscopic cholecystectomy	265	0	0	41
Abdominal				
Abdominal surgery for trauma	17	8	47	42
Emergency abdominal surgery			57	12
Splenectomy	7	0	0	43
Umbilical herniorrhaphy	24	2	8.3	6
Inguinal herniorrhaphy	1197	30	2.5	44
Bone/Joint				
Hip surgery-elective	14	0	0	45
Hip surgery-emergent	5	3	60	45
Knee surgery	42	0	0	46
Cardiac				
Cardiac surgery-elective	18	3	17	47
Cardiac surgery-emergent	5	4	80	48
Genito-urinary				
TURP	30	2	6.7	49
Hysterectomy	105	8	7.6	50
Abdominal surgeries in various liver conditions				
Acute hepatitis	11	11	100	51
Hepatitis C	34	0	0	52
Chronic hepatitis	20	0	0	36
Obstructive jaundice			5-60	53

TURP: Transurethral resection of the prostate.

(> 2.5 s above control that does not correct with vitamin K), ascites, encephalopathy, hypoalbuminemia, portal hypertension, renal insufficiency, hyponatremia, infection, anemia and malnutrition. There is no evidence suggesting that these individual risk factors are better than either CTP or MELD scores. Semi-quantitative liver function tests including galactose elimination capacity, aminopyrine breath test, indocyanine green clearance, mono-ethylglycinexylidide test (MEGX) have also been proposed to risk-stratify patients with cirrhosis undergoing surgery, but these are not available universally and hence not used routinely in clinical practice^[11].

The nature of the surgical procedure is an important determinant of postoperative complications. In general, emergency surgery is associated with a higher morbidity and mortality than elective surgery^[12-14]. The morbidity and mortality risks are highest in patients undergoing cardiac and open abdominal surgeries including cholecystectomy, gastric resection, colectomy and hepatic resection^[11]. Laparotomy causes a greater reduction in liver blood flow and increased hepatic ischemia than extra-abdominal surgery. Another contributing factor is increased risk of intra-operative bleeding in the presence of portal hypertension, especially in patients with previous abdominal surgery and adhesions.

Hemodynamic changes, characterized by increased cardiac output, splanchnic vasodilation and decreased

Table 2 Surgical severity risk stratification^[14]

Low risk	Moderate risk	High risk
Eye	Intracranial surgery	Lung resection
ENT	Laminectomy/Disc surgery	Heart surgery
Dental	Thyroidectomy	AAA repair
Sinuses/Tonsils	Other endocrine surgery	Porto-systemic shunt
Chest tube/Thoracentesis	Head and neck surgery	Splenectomy
Bronchoscopy	Major blood vessels surgery	Laparotomy
Laryngoscopy	Peripheral artery surgery	Esophagus/Stomach surgery
Tracheostomy	Embolectomy	Liver/Biliary surgery
Venous procedures	Carotid artery surgery	Small bowel/Large bowel/Pancreas surgery
Vein stripping	Rectal/Anal surgery	Renal surgery
Pacemaker insertion	Herniorrhaphy	Hip surgery
Lymph node biopsy/resection	Bladder procedures	Back fusion
GI endoscopy	Prostate procedures	Long bone fractures
Laparoscopy	Hysterectomy ± oophorectomy	
GU endoscopy	Amputations	
Male GU procedures	Hand, foot, knee surgery	
Female GU procedures	Breast biopsy/mastectomy	
D & C		
Skin lesion excision		
Superficial tumor excision		
Other diagnostic/therapeutic procedures		

ENT: Ear, nose and throat; GI: Gastrointestinal; GU: Genitourinary; D & C: Dilatation and curettage; AAA: Abdominal aortic aneurysm.

systemic vascular resistance, are common in patients with portal hypertension, and these changes progress with worsening liver disease. Despite an increased cardiac output, perfusion may be impaired due to shunting of blood, and in addition, anesthetic agents may also reduce hepatic blood flow and decrease oxygen uptake by the liver and splanchnic organs^[15,16]. Hypotension, hypoxemia, hemorrhage and use of vasoactive drugs may further reduce hepatic oxygenation. Hepatic blood flow and liver function may be further compromised by catecholamine release and other neurohormonal responses^[17].

PREOPERATIVE EVALUATION AND RISK ESTIMATION

A thorough preoperative history and physical examination are keystones in the evaluation and management of patients with chronic liver disease prior to surgery. It is essential to assess for the presence and severity of liver disease. In addition to identifying the risk factors for liver disease (blood transfusions, tattoos, illicit drug use, sexual promiscuity, family history of liver disease, alcoholism, travel history, review of prescribed or over the counter medications), it is important to elicit any previous

Table 3 Modified Child Turcotte Pugh (CTP) score

Feature	Points		
	1	2	3
Albumin (g/dL)	> 3.5	2.8-3.5	< 2.8
Prothrombin time			
-Seconds prolonged	< 4	4-6	> 6
-INR	< 1.7	1.7-2.3	> 2.3
Bilirubin (mg/dL)	< 2	2-3	> 3
(PBC/PSC) ¹	< 4	4-10	> 10
Ascites	Absent	Slight-moderate	Tense
Encephalopathy	None	Grade I - II	Grade III - IV
CTP Class	Points	2 yr patient survival (%)	
Class A	5-6	85	
Class B	7-9	60	
Class C	10-15	35	

¹In primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC), the bilirubin references are changed to reflect the fact that these diseases feature high conjugated bilirubin levels.

history of decompensation such as ascites, edema, or hepatic encephalopathy, variceal bleeding or anesthesia-related complications (Table 5). Stigmata of chronic liver disease such as jaundice, palmar erythema, spider nevi, gynecomastia, or testicular atrophy and portal hypertension such as splenomegaly, ascites, or asterixis should lead to a thorough evaluation of liver disease and its severity. Figure 1 shows general guidelines for preoperative evaluation of patients with liver disease.

A preoperative evaluation should comprise laboratory tests such as comprehensive metabolic profile, complete blood counts and prothrombin time. Elevated bilirubin, low albumin, elevated liver enzymes, low platelets and elevated prothrombin time may suggest the presence of chronic liver disease. A medical history suggestive of liver disease, clinical signs of chronic liver disease or abnormal laboratory tests should prompt further evaluation to assess severity of liver disease.

PREOPERATIVE MANAGEMENT

The outcomes of patients with cirrhosis who undergo surgery could be improved by optimizing the following premorbid conditions.

Coagulopathy

Even in urgent surgical situations, management of coagulopathy is beneficial. While peripheral vascular surgery can be performed with significant coagulopathy, neurosurgery usually requires an INR below 1.2, platelets $> 100 \times 10^9/L$, and fibrinogen $> 100 \text{ mg/dL}$. A thromboelastogram, which is a graphic representation of the interaction of plasma coagulation proteins with platelets and fibrinogen, is a useful tool to help guide the administration of coagulation factors in the operating room.

Coagulopathy in liver disease could be due to hepatic synthetic dysfunction or vitamin K deficiency usually caused by malabsorption secondary to prolonged cholestasis. Though hepatic synthetic dysfunction cannot be corrected by vitamin K administration, it may be

Table 4 Contraindications to elective surgery in patients with liver disease^[11]

Acute hepatitis	Chronic hepatitis
Alcoholic	Chronic severe hepatitis
Autoimmune	Class C cirrhosis
Uncontrolled Wilson's disease	Uncontrolled portal hypertension with sequelae
	Severe coagulopathy ¹
	Systemic liver-related co-morbidities
	Hypoxia (POPH or HPS) ²
	Cardiac volume overload
	Hepatorenal syndrome

¹Severe coagulopathy defined as platelets $< 50\,000/\text{mm}^3$ and prothrombin time prolonged beyond 3 s with no response to Vitamin K; ²POPH: Portopulmonary hypertension; HPS: Hepatopulmonary syndrome.

Table 5 American Society of Anesthesiologists (ASA) Classification of preoperative risk

ASA Class	Systemic disturbance	Mortality (%)
1	Healthy patient with no disease outside of the surgical process	< 0.03
2	Mild to moderate systemic disease caused by the surgical condition or by other pathological processes, medically well-controlled	0.2
3	Severe disease process which limits activity but is not incapacitating	1.2
4	Severe incapacitating disease process that is a constant threat to life	8
5	Moribund patient not expected to survive 24 h with or without an operation	34
E	Suffix to indicate emergency surgery for any class	Increased

^b $P < 0.01$ vs group B and control group; ^a $P < 0.05$ vs control group; ^d $P < 0.01$ vs group B and control.

worthwhile to administer a trial of parenteral vitamin K in elective surgery cases in case malabsorption is a contributing factor. In emergency situations or cases in which there is inadequate response to vitamin K, fresh frozen plasma can be administered. If patients do not respond to fresh frozen plasma, Cryoprecipitate, which contains large amount of fibrinogen and von Willebrand factor, may be given intravenously preoperatively. DDAVP $0.3 \mu\text{g/kg}$, which contains endogenous von Willebrand factor and factor VIIa infusion are other novel agents that could be used to correct clotting abnormalities. In most refractory cases, plasma exchange may be necessary. In addition to the correction of coagulopathy, prophylactic platelet transfusions may be considered for severe thrombocytopenia ($< 50\,000/\text{mL}$).

Ascites

The presence of ascites may increase the risk of abdominal wound dehiscence, abdominal wall herniation, and respiratory compromise. Ascites could be managed by a combination of low sodium diet and administration of diuretics such as furosemide and spironolactone with careful monitoring of creatinine and electrolyte levels. If ascites is uncontrolled prior to surgery, large volume

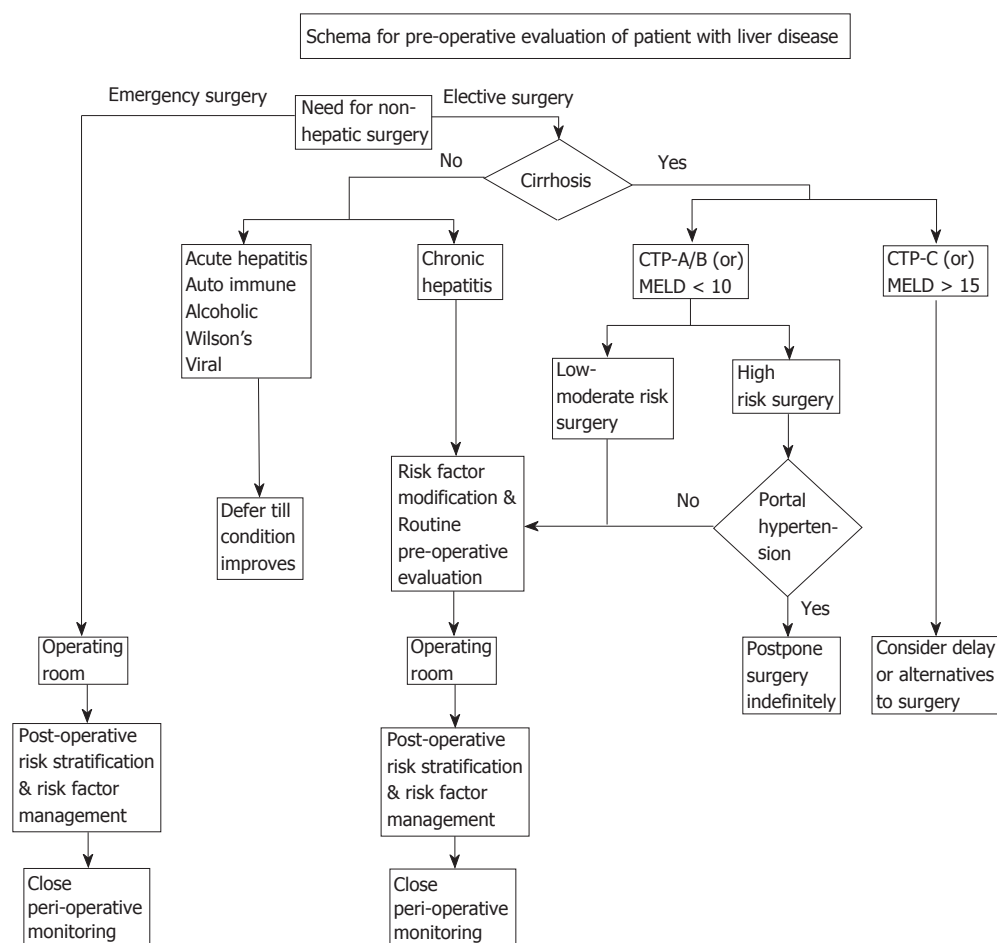


Figure 1 An algorithm for evaluation and management of patients with liver disease prior to surgery.

paracentesis could be performed either preoperatively or intra operatively. Replacement with albumin (8 g for each liter of ascites drained) is important to maintain intravascular volume and to reduce the risk of hepatorenal syndrome.

Patients with ascites could present with abdominal pain from spontaneous bacterial peritonitis (SBP) or secondary bacterial peritonitis. In such situations, it is important to analyze the ascites fluid for differential white cell counts. If absolute ascites neutrophil counts exceed 250 neutrophils/mL, the patient should be treated for SBP with a third generation cephalosporin such as ceftriaxone or a fluoroquinolone such as ciprofloxacin as the common pathogens are usually *Escherichia coli* and *Klebsiella pneumoniae*. If multiple organisms are grown, secondary bacterial peritonitis should be suspected.

Since ascites may reaccumulate rapidly with administration of normal saline, it is advisable to use colloids or blood perioperatively. The role of pre-operative transjugular portosystemic shunt (TIPS) to control refractory ascites is poorly defined and is not currently recommended^[18].

Renal dysfunction

In advanced liver disease, patients may present with renal insufficiency from a variety of etiologies including iatrogenic causes such as medications [diuretics, non-steroidal anti-inflammatory drugs (NSAID) or other nephrotoxic agents] or large volume paracentesis (often performed without albumin replacement), infections (SBP and urinary tract infections are most common), gastrointestinal bleeding

or hepatorenal syndrome (HRS). HRS occurs in the setting of advanced portal hypertension and associated profound splanchnic vasodilation. These changes result in a cascade of events leading to decreased renal perfusion and renal insufficiency. From a practical point of view, HRS is defined by a plasma creatinine > 1.5 mg/dL, in the absence of any iatrogenic causes, bleeding or intrinsic renal diseases, that is not improved by fluid (1.5 liters of isotonic saline) or colloids (albumin 1 g/kg body weight for 3 d) administration^[19]. HRS may present in two different ways; rapidly progressive (type 1) where creatinine doubles (100% increase) over a period of 2 wk and slowly progressive (type II) where creatinine is ≥ 1.5 mg/dL, and then progresses slowly. Type 1 HRS has a dismal outcome without liver transplantation. However, HRS that is precipitated by SBP and the intravascular fluid shifts associated with anesthesia or surgery is potentially reversible. Combination treatments with midodrine and octreotide, or terlipressin and albumin have shown promising results, but there is a paucity of controlled data with adequate sample size on these treatment modalities^[20,21]. Hemodialysis or continuous veno-venous hemodialysis (CVVHD) is often used as a bridge to liver transplantation; again there are no controlled studies proving its efficacy.

Hyponatremia

Hyponatremia is common in advanced liver disease. Severe hyponatremia may lead to seizures and worsening of hepatic encephalopathy. Rapid correction (> 10 meq/L per 24 h) of sodium, especially in malnourished patients, could

lead to fatal central pontine myelinolysis. Hyponatremia is usually corrected by fluid restriction ($< 1000 \text{ mL/d}$) and discontinuation of all diuretics. In symptomatic patients, intravenous 3% sodium chloride may be used judiciously^[22].

Hepatic encephalopathy

It is important to recognize sub-clinical encephalopathy preoperatively. Many conditions including constipation, alkalosis, central nervous system depressants, hypoxia, infection, azotemia and gastrointestinal bleeding may precipitate overt **hepatic encephalopathy (HE)**. In patients with cirrhosis, especially in those with clinical or sub-clinical HE, many of these precipitating factors could be avoided or treated early to prevent significant HE. HE may complicate the post-operative course resulting in immobility, lack of cooperation with nursing procedures, unnecessary investigations (if not diagnosed clinically or suspected) and aspiration pneumonia. Encephalopathy is treated with lactulose 30 mL orally every 6 h, titrated to 2 or 3 soft bowel movements daily. Metronidazole, rifaximin and low dose neomycin could be used in combination with lactulose, but it is important to avoid neomycin in patients with renal insufficiency^[23-25].

Malnutrition

Malnutrition can result in hypoalbuminemia, low oncotic pressure and intravascular hypovolemia. Muscle wasting may result in patient immobility and respiratory muscle dysfunction, leading to prolonged mechanical ventilation postoperatively. When hospitalized with malnutrition, a nutritionist consultation should be obtained. Patients with advanced liver disease should receive nutritional supplementation, both enteral and parenteral in the perioperative period. Consideration should be given to starting the supplementation preoperatively, and this may reduce the short-term mortality and postoperative complications, but the impact of nutritional supplementation on long-term mortality remains unclear^[26,27]. The supplementation should be high in carbohydrate/lipid content and low in amino acid content to prevent worsening of preexisting hepatic encephalopathy. Nutritional supplementation is particularly important in alcoholics and should include vitamin B1.

Pulmonary conditions

Common conditions include hydrothorax, hepatopulmonary syndrome, portopulmonary hypertension, immune mediated lung diseases (especially in autoimmune diseases) and emphysema (in smokers and alpha-1 antitrypsin deficiency). Hepatic hydrothorax occurs, usually on the right side, in about 5% of patients with cirrhosis. Preoperative thoracentesis is not usually recommended as the resulting hypoxemia is not very severe^[7]. Use of an incentive spirometer postoperatively may prevent atelectasis. Hepatopulmonary syndrome includes the presence of systemic-to-pulmonary vascular shunts and intrapulmonary arteriovenous shunts, both of which result in systemic arterial desaturation. It is marked by orthodeoxia and platypnea and can be diagnosed by contrast

echocardiography or a Technetium 99 m-labelled albumin scan. This could be further confirmed by pulmonary angiography. **Type 1 HPS is a pattern, which responds well to 100% oxygen supplementation.** Type 2 HPS does not respond to oxygen and is considered a contraindication to general anesthesia. Portopulmonary hypertension occurs in 2%-4% of **patients with cirrhosis and portal hypertension.** It is defined as a mean pulmonary artery pressure (MPAP) greater than 24 mmHg with a normal pulmonary capillary wedge pressure and a pulmonary vascular resistance greater than 120 dynes/s per cm^5 . When the MPAP is $> 50 \text{ mmHg}$, the condition becomes life threatening and can lead to right ventricular failure and hypoxemia. If surgery is contemplated, pulmonary pressures must be optimized usually with intravenous epoprostenol^[28], but sildenafil or bosentan have also been tried perioperatively.

Cardiac conditions

Dobutamine stress echocardiography has been advocated as a screening modality for coronary artery disease in patients with cirrhosis. However, its predictive value in patients with advanced cirrhosis is quite variable. In general, the **American College of Cardiology and American Heart Association** guidelines are useful to assess the suitability of patients with liver disease for non-cardiac surgery. Beta-blockers must be used in the perioperative period if no contraindications exist. They are not only useful in reducing the incidence of perioperative myocardial ischemia and adverse cardiac events, but also help to decrease the portal pressure. In patients with advanced cirrhosis and cardiac disease, less invasive procedures such as angioplasty, valvuloplasty and newer off-pump procedures may prove to be beneficial^[29,30].

Miscellaneous

Anemia must be managed aggressively with early and sufficient substitution of clotting factors and blood products. Glucose intolerance and diabetes are common in patients with liver disease. Insulin infusion is beneficial to maintain good control perioperatively. However, caution is warranted as patients with cirrhosis are also at risk for hypoglycemia^[31]. Deficiency in 25-hydroxyvitamin D can occur in cirrhosis and if associated with nutritional deficiencies will lead to osteomalacia and will require Vitamin D and Calcitriol supplementation. Screening for varices should be performed in cirrhotic patients and those receiving beta blockade for prophylaxis of variceal bleeding and **should continue to do so in the perioperative period**^[32,33]. Preoperative antibiotic prophylaxis is mandatory due to high rates of septic complications in patients with cirrhosis. In an emergency situation, especially after resectional procedures, selective bowel decontamination and prolonged course of antibiotics must be considered. In patients with peptic ulcer disease, long-term maintenance therapy with proton pump inhibitors may be recommended^[34].

Strategies for specific liver conditions

Stress-dose steroids should be administered preoperatively in patients with autoimmune hepatitis who are on steroid

therapy. Patients treated with D-pencillamine for Wilson's disease can suffer from poor wound healing and thereby, the dosage should be decreased preoperatively and for one to two weeks postoperatively.

OPERATIVE AND POSTOPERATIVE MANAGEMENT

Liver dysfunction may result in prolonged duration of action of anesthetic and neuromuscular blocking agents because of altered metabolism or clearance rates. Isoflurane is the preferred anesthetic agent for patients with cirrhosis, while methoxyflurane, chloroform and halothane should be avoided if possible. In addition, the actions of neuromuscular blocking agents may be prolonged due to increased biliary excretion and decreased pseudocholinesterase activity. Therefore, atracurium is the drug of choice in patients with liver disease or biliary obstruction, and doxacurium is recommended for prolonged surgeries. Oxazepam and lorazepam are the most suitable anxiolytic sedatives, whereas fentanyl and sufentanyl should be the first-line narcotics. In contrast, morphine, meperidine and barbiturates can precipitate hepatic encephalopathy and should be avoided^[35].

Postoperative complications in patients undergoing surgery include (1) **cirrhosis related complications**: worsening or new onset ascites, worsening or new onset hepatic encephalopathy (Grade 1-4), upper gastrointestinal bleeding, worsening or new onset acute renal failure/new need for dialysis, hepatorenal syndrome (acute renal failure in the setting of decreasing liver function with no other clinical, laboratory or anatomic cause for the renal failure), liver failure, and coagulopathy [disseminated intravascular coagulation (DIC), increased prothrombin time, increased activated partial thromboplastin time, decreased fibrinogen concentration, decreased platelet count]; (2) surgical wound complications: infection, dehiscence, eventration, fistula, abscess, surgical site bleeding; and (3) general complications: pneumonia/Acute Respiratory Distress Syndrome (ARDS), ventilation dependence, chronic obstructive pulmonary diseases (COPD) exacerbation, chronic heart failure/arrhythmia/myocardial infarction, urinary tract infections, paralytic ileus, phlebitis/PE, and death.

The common complications of surgery in patients with cirrhosis are hemorrhage, sepsis, liver failure, fluid overload and hepatorenal syndrome. Patients with cholestatic jaundice are at an increased risk of postoperative renal failure. Increased postoperative bilirubin levels (> 3 mg/dL), creatinine levels and a decreased albumin level are associated with greater mortality^[36].

Pre-operative strategies, which has been discussed earlier to minimize complications, should be continued in the post-transplant period. In addition, in the post-operative period, one needs to closely monitor fluid balance and nutrition, identify and correct electrolyte abnormalities, coagulopathy, encephalopathy and infection. Non-steroidal anti-inflammatory drugs and nephrotoxic drugs should be avoided, and caution should be exercised with use of narcotics.

CONCLUSION

The mortality from abdominal surgeries in patients with cirrhosis is unacceptably high. Preoperative assessment can predict survival to some extent in cirrhotic patients requiring abdominal procedures. Furthermore, optimization of premorbid factors may reduce perioperative mortality and morbidity. However, better predictive models for risk-stratification are necessary to predict and improve post-operative outcomes of patients with cirrhosis^[37].

ACKNOWLEDGMENTS

We thank Murtuza Balasinorwala for his help with the preparation of the illustrations.

COMMENTS

Background

In patients with cirrhosis, the surgical mortality is as high as 25%. Pre-operative risk stratification in such patients and optimization of medical care prior to surgery are essential to prevent adverse outcomes after surgery.

Research frontiers

Studies with better predictive models for risk-stratification are necessary to predict and improve post-operative outcomes of patients with cirrhosis.

Innovations and breakthroughs

The recent adoption of the MELD score by UNOS to risk stratify patients prior to transplant surgery has improved our ability to better characterize the severity of liver disease objectively in liver transplant population. Similar models are necessary to risk-stratify patients prior to any other surgery in patients with cirrhosis.

Applications

CTP score, MELD score, age, and American Society of Anesthesiologists class are factors that can quantify the risk of mortality postoperatively in patients with cirrhosis, independent of the procedure performed. These factors can be used in determining operative mortality risk, and to decide whether elective surgical procedures can be delayed until after liver transplantation.

Terminology

MELD score is a numerical score obtained by inserting the values of serum total bilirubin, INR and serum creatinine into a logarithmic formula. It is a disease severity index score and helps determine the mortality without liver transplantation in patients with advanced cirrhosis.

Peer review

The authors reviewed the pathophysiology, morbidity and mortality associated with non-hepatic surgery in patients with cirrhosis, and then recommend an algorithm for risk assessment and evidence based management strategy to optimize post-surgical outcomes. They concluded that better predictive models for risk-stratification are necessary to predict and improve post-operative outcomes of patients with cirrhosis

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S- Editor Zhu LH L-Editor Zhu LH E-Editor Li JL

TOPIC HIGHLIGHT

Paul Joseph Thuluvath, Professor, Series Editor

Pancreatic sphincterotomy: Technique, indications, and complications

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Received: 2007-05-16 Accepted: 2007-05-28

Abstract

Pancreatic sphincterotomy serves as the cornerstone of endoscopic therapy of the pancreas. Historically, its indications have been less well-defined than those of endoscopic biliary sphincterotomy, yet it plays a definite and useful role in diseases such as chronic pancreatitis and pancreatic-type sphincter of Oddi dysfunction. In the appropriate setting, it may be used as a single therapeutic maneuver, or in conjunction with other endoscopic techniques such as pancreatic stone extraction or stent placement. The current standard of practice utilizes two different methods of performing pancreatic sphincterotomy: a pull-type sphincterotome technique without prior stent placement, and a needle-knife sphincterotome technique over an existing stent. The complications associated with pancreatic sphincterotomy are many, although acute pancreatitis appears to be the most common and the most serious of the early complications. As such, it continues to be reserved for those endoscopists who perform a relatively high-volume of therapeutic pancreaticobiliary endoscopic retrograde cholangio-pancreatography.

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Key words: Pancreas; Sphincterotomy; Endoscopic; Technique; Indications; Complications

Buscaglia JM, Kalloo AN. Pancreatic sphincterotomy: Technique, indications, and complications. *World J Gastroenterol* 2007; 13(30): 4064-4071

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INTRODUCTION

Since its initial application in 1974, endoscopic biliary

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sphincterotomy has revolutionized the approach to patients with biliary tract diseases^[1]. Utilizing biliary sphincterotomy in conjunction with stone extraction, stent placement, or stricture dilatation has become the standard of care for problems that were once only remedied by surgical procedures. Endoscopic therapy for pancreatic disorders has not advanced quite so rapidly, however^[2]. Pancreatitis and its associated complications have prevented some endoscopists in the past from attempting to apply similar therapeutic techniques as those used in treating biliary tract disorders. In addition, clear-cut indications for endoscopic therapy of the pancreas have been much more difficult to define due to a paucity of well-designed clinical trials justifying its use. Most of the techniques that have been used in previous studies were performed on small numbers of patients, and in expert centers only. The majority of studies have been retrospective in nature, lacking randomization with a prospective design^[2].

It is on this background in which the topic of endoscopic pancreatic sphincterotomy (EPS) is reviewed. EPS is the cornerstone of endoscopic therapy^[3] of the pancreas, and once access is obtained, EPS may be used as a single therapeutic maneuver (e.g. to treat pancreatic-type sphincter of Oddi dysfunction), or in series with other endoscopic therapeutic techniques such as stone extraction or stent placement^[1,4]. The following review will attempt to provide an evidence-based summary of the technique, the indications, and the complications associated with endoscopic pancreatic sphincterotomy.

THE ENDOSCOPIC TECHNIQUE

The main principles involved in EPS are very much like those of biliary sphincterotomy. They involve wire-guided cannulation of the duct prior to cutting, and they utilize a slow and stepwise approach that relies on accurate identification of anatomical landmarks. There are essentially two different types of techniques that are used by most expert endoscopists when performing this procedure. The first approach, and the more widely utilized, is performed while using a standard pull-type sphincterotome. The second approach uses an endoscopic needle-knife to cut the sphincter muscle after placement of a pancreatic duct stent. Both techniques have their advantages and disadvantages, and the details surrounding each approach are discussed here. In addition, pre-cut or 'access' pancreatic sphincterotomy in those instances when the endoscopist is faced with a difficult pancreatic

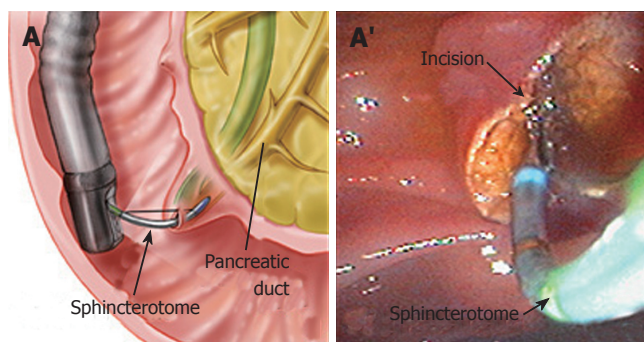


Figure 1 EPS performed using a pull-type sphincterotome without prior pancreatic stent placement.

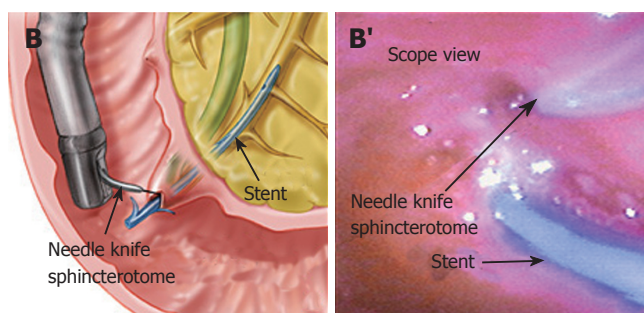


Figure 2 EPS performed with a needle-knife sphincterotome over a pancreatic stent.

annulation will be discussed. Finally, sphincterotomy of the minor papilla will be briefly mentioned as well.

PULL-TYPE SPHINCTEROTOMY

Once successful cannulation of the pancreatic duct orifice is achieved, the guidewire is advanced into the main pancreatic duct and confirmation of position is usually obtained with a contrast pancreatogram. Assuming a clear indication for sphincterotomy has been established, this part of the procedure is most often performed with a pull-type sphincterotome. Like biliary sphincterotomy, the incision should be 'hot and slow'^[5]. It should be directed towards the 1 to 2 o'clock position with the very distal part of the cutting wire^[6-8]. In other words, most of the cutting wire should be visible outside the papillary orifice. Note that the direction of the cut is very different from that of a biliary sphincterotomy. In biliary sphincterotomy, the cutting direction is in the 11 o'clock to 1 o'clock position (preferably the 12 o'clock position). The sphincterotome is slightly bowed while the cutting wire is 'walked up' the roof of the papilla in a stepwise fashion^[6]. In pancreatic sphincterotomy, the same principles apply, but the direction is more towards the right, guiding the cutting wire along the floor of the papillary orifice (Figure 1).

The actual incision should be performed using the pure cutting current with the electrosurgical generator^[7]. This prevents further damage to the pancreas and limits the possible future development of fibrosis and papillary stenosis^[8-10]. The length of the cut is generally between 5 mm and 10 mm. Larger diameter ducts require longer

cuts in order to achieve the largest possible access. Once the sphincterotomy has been completed, a temporary pancreatic stent is usually left in place for a short period of time in order to help facilitate adequate drainage from the duct. The edema that ensues following a pancreatic sphincterotomy can cause ductal obstruction and eventual pancreatitis^[11]. This policy of placing a pancreatic stent after every pancreatic sphincterotomy, however, is not universal. Some expert endoscopists do not feel the need to perform this step. Moreover, the types of stents that are chosen and the desired duration of use are also debated^[12].

Early in the era of pancreatic sphincterotomy, many endoscopists advocated always performing EPS in concert with a prior biliary sphincterotomy. Biliary sphincterotomy done immediately before pancreatic sphincterotomy is felt by some to allow for easier identification of clear anatomical landmarks, thus making it a safer and more effective procedure. It may provide better exposure of the pancreaticobiliary septum, and therefore allow improved access to the desired pancreatic tissue^[13]. Also, this method of choice prevents the rare possibility of biliary complications following a primary pancreatic cut^[1]. This includes inadvertent damage to the distal bile duct, as well as possible biliary obstruction due to edema adjacent to the biliary duct orifice. Many recommend a biliary sphincterotomy before a pancreatic sphincterotomy in cases of cholangitis or obstructive jaundice, a common bile duct diameter > 12 mm, or an alkaline phosphatase level > twice normal^[8]. It may also be performed when there is a need to obtain improved access to the main pancreatic duct^[14].

NEEDLE-KNIFE SPHINCTEROTOMY

An alternative method to pancreatic sphincterotomy utilizes an endoscopic needle-knife instead of a standard pull-type sphincterotome. Cutting with the needle-knife is done only after placement of a pancreatic duct stent. The tip of the needle-knife is placed at the most proximal portion of pancreatic sphincter tissue that is overlying the stent. While using the stent as a guide to direct the cut along the plane of the pancreatic duct, the needle-knife tip is advanced over the top of the stent and down its longitudinal axis thereby 'unroofing' the intraduodenal portion of the major papilla (Figure 2). Incision length is similar to that of sphincterotomy with a pull-type sphincterotome; that is, the length is generally between 5 mm and 10 mm. Many experts believe that a prior biliary sphincterotomy is especially helpful before utilizing needle-knife technique^[13]. Good exposure of the pancreaticobiliary septum allows for better tissue access and more effective 'septotomy'.

There are a few limitations to this technique, however. The absolute prerequisite of pancreatic stent insertion makes it a technique that may not always be feasible if a stent cannot be placed. For example, in chronic pancreatitis, it may be very difficult to insert a stent without first removing ductal calculi^[8]. Also, many endoscopists find it easier to perform the sphincterotomy without having to first exchange the sphincterotome, place the stent, and then insert the needle-knife in order to

perform the cut. Finally, most biliary sphincterotomies are performed with a standard pull-type sphincterotome, and so many are accustomed to performing EPS in a similar fashion. Furthermore, many experts may argue that EPS could be done in a more controlled fashion using this technique

Despite the fact that pancreatic sphincterotomy is performed by only two different techniques, survey questionnaires show that there is a lack of expert consensus in terms of which is the better approach. A recent survey of 14 expert endoscopists in nine US centers showed that six of the 14 gastroenterologists either 'always' or 'often' use the pull-type sphincterotome technique, while seven out of 14 'always' or 'often' use the needle-knife technique^[12]. Eight physicians 'always' perform a biliary sphincterotomy prior to pancreatic sphincterotomy, and only two of 14 use pure cutting current during the procedure. Almost all endoscopists insert a pancreatic stent after sphincterotomy, as it lowers the likelihood of post-ERCP pancreatitis^[11]. However, which types of stents to be used and how long to leave them in place for is quite variable among those who perform EPS on a regular basis^[12].

PRE-CUT PANCREATIC SPHINCTEROTOMY

The pre-cut pancreatic sphincterotomy refers to an endoscopic technique that allows access to the pancreatic duct without performing prior deep cannulation. It is usually done when access to the duct is blocked in some manner (e.g. an impacted stone)^[9,15]. Once the pancreatic duct is accessed, conventional pancreatic sphincterotomy is performed. Generally, this technique is not utilized as often as the pre-cut biliary sphincterotomy since a difficult pancreatic duct cannulation is encountered far less often than a difficult biliary cannulation. The pancreatic pre-cut is done in a manner that is very similar to the biliary pre-cut sphincterotomy. Most endoscopists will use a free-hand needle-knife to perform the pre-cut, although there are several options for this technique^[15]. In the case of a stone that is obstructing the pancreatic orifice, for example, a needle-knife can be used to cut the papillary mucosa lying directly over the stone. Once the stone is released and the obstruction is relieved, the pancreatic duct can be cannulated in the usual manner to prepare for a conventional pancreatic sphincterotomy.

MINOR PAPILLA SPHINCTEROTOMY

Minor papilla sphincterotomy is actually a misnomer since the cutting that is performed during this technique is more of a 'papillotomy', rather than a true muscle 'sphincterotomy', per se. Hence, the term is more appropriately phrased as 'minor papillotomy'. It was first described by Cotton in 1978 as a means to treat recurrent dorsal pancreatitis^[16]. Since then, it has successfully emerged as a recognized and effective treatment for patients with pancreas divisum who require ductal decompression. Like sphincterotomy of the major papilla, minor papillotomy can be performed using two different techniques: standard pull-type technique and needle-knife technique. The pull-type technique involves wire-guided cannulation of the dorsal duct with a regular size

papillotome. Although some advocate the use of an ultra-tapered tip papillotome in this setting (e.g. 3-F), we find a regular papillotome to be softer on the papilla and allow for easier cannulation. In addition, a soft-tipped, hydrophilic, 0.035-inch guidewire is generally used during cannulation. Once deep cannulation is achieved, the papillotome may be slightly bowed while the cutting wire is directed along the course of the dorsal duct (usually in the 11 o'clock position) so as to completely ablate the mucosal mound of minor papilla. Either pure cutting current or blended current on the electrosurgical generator may be used.

The needle-knife technique is similar to that of EPS of the major papilla. Following wire-guided cannulation, a small diameter 3-F or 5-F pancreatic stent is first placed over the wire and through the minor papillary orifice into the proximal dorsal duct. Once the stent is in position and the guidewire is removed, a needle-knife is used to cut the portion of the minor papillary mound above the stent. The needle-knife cutting wire is generally directed in the 11 o'clock position along the course of the dorsal duct as the minor papilla is 'unroofed'. Again, either blended current or pure cutting current may be used.

INDICATIONS FOR EPS

Unlike endoscopic biliary sphincterotomy, literature that describes and validates the indications for pancreatic sphincterotomy is sparse. There are several reasons for this disparity. First, EPS appears to be mainly performed at specialized referral centers. Physicians performing this procedure usually have years of experience in therapeutic biliary and pancreatic endoscopy. In order to perform EPS with adequate proficiency, the endoscopist must typically practice in an environment that yields a relatively high volume of ERCP. The centers that perform EPS should be capable of handling all the possible complications associated with this procedure. Furthermore, it is the relatively high likelihood of complications seen with EPS that contributes to the reluctance among physicians to perform this technique. As a result, there have been fewer published studies over the years that outline the indications, outcomes, and safety of pancreatic sphincterotomy.

EPS may be indicated for a variety of diseases and disease-related manifestations that involve the pancreas. In general, it is easier to consider the indications for EPS in terms of primary or secondary therapy (Table 1). In other words, this technique may be performed by itself as the primary treatment modality (i.e. for the treatment of pancreatic-type sphincter of Oddi dysfunction); or it may be utilized as a secondary treatment modality in facilitating a further intervention (i.e. better access to the main pancreatic duct before dilating a downstream dominant stricture. Overall, there is far more data available regarding the use of EPS in conjunction with an additional intervention (secondary therapy) than for using this technique alone (primary therapy)^[4].

EPS AS PRIMARY THERAPY

Most of the literature describing pancreatic sphincterotomy as the primary endoscopic therapy of choice is concentrated

Table 1 Indications for endoscopic pancreatic sphincterotomy (EPS)

EPS as primary therapy
Sphincter of Oddi dysfunction (SOD)
-Pancreatic SOD
-Biliary SOD unresponsive to biliary sphincterotomy
Chronic pancreatitis with papillary stenosis/stricture
Pancreas divisum (EPS of the minor papilla)
EPS to facilitate a further intervention
Chronic pancreatitis treated with pancreatic stent and/or stone removal
Pancreatic pseudocyst treated with transpapillary drainage
Resection of an ampullary adenoma
Pancreatic fistula treated with stent placement
Pancreatic disease due to malignancy
-Primary pancreatic cancer causing strictures, stones, pseudocysts
-Metastatic disease to the pancreas causing strictures, stones,

on the area of pancreas divisum and minor papillotomy. This is a separate topic and should be reserved for a separate review. However, EPS has been shown to provide primary therapeutic benefit in patients with at least two separate and distinct disorders: pancreatic-type sphincter of Oddi dysfunction (SOD) and chronic pancreatitis.

SOD is a benign obstruction to the flow of bile or pancreatic juice at the level of the pancreaticobiliary junction^[16-20]. It is due to functional dyskinesia or hypertension of the biliary and/or pancreatic portion of the sphincter^[21-25]. It results in transient noncalculous obstruction, causing abdominal pain or pancreatitis^[26-30]. Isolated pancreatic-type SOD may be seen in 15% to 20% of all patients with acute recurrent pancreatitis of unknown etiology. It has been estimated to occur in 25% of all patients undergoing manometry for suspected SOD^[31-35]. Type 1 pancreatic SOD is characterized by the triad of pancreatic-type abdominal pain, elevated amylase and lipase levels, and a dilated main pancreatic duct^[36,37]. Type 2 pancreatic SOD has pancreatic-type abdominal pain associated with either elevated enzyme levels or a dilated duct^[16].

The overall clinical response rate of endoscopic sphincterotomy for SOD (biliary and pancreatic) ranges between 55% and 95%. Patients with Type 1 pancreatic SOD are most likely to benefit from EPS. Several studies have shown that these patients may experience a significant reduction in pain and clinical episodes of pancreatitis. Type 2 pancreatic SOD may also achieve benefit from EPS, but some prefer to document abnormal pancreatic manometry before undergoing sphincterotomy. In addition, more recent studies have suggested a clinical benefit from EPS in those patients who have persistent pain despite prior biliary sphincterotomy^[38].

A pancreatic sphincterotomy alone is frequently used as the primary treatment modality in moderate to severe chronic pancreatitis. The rationale for treating chronic pancreatitis with endoscopic therapy is based on the principle of decreasing pancreatic intraductal pressure. In moderate to severe disease, the development of ductal stones, protein plugs, and ductal strictures may occur. Each of these can cause partial or complete obstruction to the flow of pancreatic juice out into the duodenum, resulting in permanent alterations to the duct morphology. Ductal obstruction leads to tissue hypertension, and thus tissue

ischemia. Karanjia *et al*^[39] demonstrated a reduction of pancreatic blood flow after ligation of the main pancreatic duct (therefore producing intraductal hypertension) in a feline model of pancreatitis. The reduction of blood flow was partially reversed after relief of the main duct obstruction. It is believed that the symptom of pain in chronic pancreatitis is directly due to this parenchymal ischemia^[1].

Another consequence of obstruction to the main pancreatic duct is secondary obstruction to the smaller side branch ducts. This ultimately causes parenchymal atrophy. As the tissue begins to atrophy, the pancreas loses its ability to perform both its endocrine and exocrine functions. A therapeutic intervention that could minimize intraductal pressure might help to prevent this dangerous cascade of events, thus diminishing pain and preserving function. This is the basis, although controversial, behind EPS in chronic pancreatitis.

Few studies have specifically examined the role of EPS as the sole endoscopic therapy in chronic pancreatitis. Most studies that have investigated this topic have done so in the context of additional endoscopic interventions. Studies like this need to be examined closely in order to separate those patients who received EPS alone versus those who received EPS in concert with an additional endoscopic technique. This is often difficult, especially if the authors have not clearly distinguished between the two groups. Nonetheless, several studies have attempted to evaluate the safety and long-term results of pancreatic sphincterotomy in chronic pancreatitis.

Ell *et al*^[40] described pancreatic sphincterotomy in 118 patients with chronic pancreatitis. Eighty percent of the patients underwent a standard pull-type sphincterotomy, while 20% underwent a needle-knife technique. Overall, 98% of the sphincterotomies performed were successful, and the complication rate was only 4.2%, including four cases of moderate pancreatitis and one case of severe bleeding. The results in terms of pain relief were not examined in this study, however.

Okolo *et al*^[41] retrospectively analyzed 55 patients who had a pancreatic sphincterotomy. Forty patients (73%) underwent the procedure for the indication of symptomatic chronic pancreatitis. The goal of the study was to assess the long-term efficacy of sphincterotomy with pain relief being the primary endpoint. After a median follow-up of 16 mo, 60% of all patients reported a significant improvement in their pain scores.

Papillary stenosis appears to be a clear-cut indication EPS in those patients with symptomatic chronic pancreatitis. Without significant ductal abnormalities distal to the papilla, pancreatic sphincterotomy by itself can be confidently utilized as the primary endoscopic therapy of choice in these patients. Similarly, mucinous ductal ectasia involving the proximal main pancreatic duct is also a proven indication for EPS in those patients with recurrent pancreatitis^[4].

EPS AS SECONDARY THERAPY

Pancreatic sphincterotomy is commonly performed in concert with other endoscopic techniques such as stent placement or balloon dilatation of the pancreatic duct.

Table 2 Complications of endoscopic pancreatic sphincterotomy (EPS)

Early Complications (< 3 mo, typically < 72 h)

Pancreatitis
Severe bleeding
Perforation
Pancreatic and/or biliary sepsis

Late Complications (> 3 mo)

Papillary stenosis
Proximal pancreatic duct strictures

Stent-related Complications (variable timing)

Ductal and parenchymal changes
Stone formation
Infection
Ductal perforation
Stent migration
Stent occlusion
Duodenal erosion

In this setting, the purpose of the sphincterotomy is to help facilitate the primary therapy (i.e. removal of stones from the duct or dilatation of a ductal stricture). There are several diseases and conditions in which EPS is used in this manner (Table 1). The decision to cut the sphincter in these situations is based on sound clinical judgment by the endoscopist, and whether or not he or she feels that the risk of EPS is outweighed by the potential benefit gained in aiding the primary therapy.

In moderate to severe chronic pancreatitis, ductal strictures and stones are frequently encountered. If their location within the main duct is very distal to the papilla, EPS alone may not be sufficient. Stone removal or stricture dilatation is often times the main goal of ERCP for certain patients. Pancreatic sphincterotomy may be needed before the procedure for better access to the duct (precut), or it can be used simply to help reduce intraductal hypertension and allow for easier flow of juice and calculous debris out into the duodenum. This also holds true, for example, when treating pancreatic pseudocysts by means of a transpapillary approach. For those pseudocysts that communicate with the main pancreatic duct, a stent is placed within the duct in order to bridge the fistulous connection^[42]. EPS in this setting also helps to reduce intraductal pressures and facilitate flow out towards the papilla.

Other clinical scenarios for which sphincterotomy is indicated as secondary therapy include stent placement prior to surgery for mucinous ductal ectasia, as well as stent placement in the treatment of a pancreatic fistula^[4]. EPS may also be used in concert with a pancreatic stent following the resection of an ampullary adenoma. Here, the purpose of the sphincterotomy (and the stent) is to reduce the risk of post-procedural pancreatitis due to peri-ampullary edema. Finally, sphincterotomy is often indicated for the palliative treatment of strictures, stones, and pseudocysts in malignant obstruction of the pancreas.

COMPLICATIONS OF EPS

In general, there are essentially three different types of complications associated with pancreatic sphincterotomy: early, late, and stent-related complications (Table 2)^[43].

Early complications are usually recognized within the first 72 h after the procedure, but often times within the first few hours. They include pancreatitis, severe bleeding, perforation, and pancreatic or biliary sepsis. Late complications are encountered at least 3 mo after the procedure, and this category mainly consists of papillary stenosis and proximal ductal strictures. Stent-related complications include pancreatic ductal and parenchymal changes, stone formation, infection, ductal perforation, stent migration, stent occlusion (causing pain and/or pancreatitis), and duodenal erosion.

Within the last 13 years, there have been four major studies that have examined the rates of complication associated with pancreatic sphincterotomy of the major papilla^[14,44-46]. In a study by Kozarek *et al*^[13], 56 patients underwent EPS. Fifty-four (96%) patients had chronic pancreatitis and two patients had acute recurrent pancreatitis. The indications for the sphincterotomy were as follows: obstructing ductal calculi (26), ductal disruption and leak (12), sphincter stenosis (10), and dominant stricture (8)^[14]. Forty-seven patients had a pull-type sphincterotomy, and 33 of these patients also had a pancreatic stent placed after the sphincterotomy. Nine patients had a needle-knife sphincterotomy over an existing pancreatic stent. Early complications occurred in 10.7% of the patients, and they included pancreatitis (4 patients, 7.1%) and cholangitis (2 patients, 3.6%). Late complications, however, occurred in 30% of the patients: 14% with papillary stenosis, and 16% with asymptomatic ductal changes (thought to be due to the stent placement).

Esber *et al*^[44] reported the complications of EPS in 236 consecutive patients. A pull-type sphincterotomy was performed in 123, and 87 patients in this group also had a stent placed following the sphincterotomy. Needle-knife sphincterotomy over a pancreatic stent was performed in 113 patients. Seventy-four percent of the patients had a sphincterotomy for the purposes of treating pancreatic-type SOD, while 26% had chronic pancreatitis and the procedure was performed to facilitate an additional endoscopic maneuver such as removal of stones, stricture biopsy, *etc*. Overall, post-ERCP pancreatitis occurred in 14% (mild in 76%, moderate in 21%, and severe in 3%). Other various complications occurred in only 1.7% of the cases. The rate of pancreatitis was 15.5% in the patients with pancreatic-type SOD. It was only 9.7% in the patients with chronic pancreatitis. It has been suggested that the reason for this lower rate of post-ERCP pancreatitis is due to the periductal fibrosis and scarring seen in those patients with underlying chronic pancreatitis. In other words, the limited amount of nearby healthy pancreatic parenchyma offers some protection against the injury that occurs after a pancreatic sphincterotomy^[14,43].

Parsons *et al*^[45] evaluated the complication rate of performing a stentless pancreatic sphincterotomy. In 31 patients, EPS was done with a pull-type sphincterotome followed by the placement of a nasopancreatic tube. All the tubes were removed within 24 h of placement. Post-ERCP pancreatitis was observed in one patient (3.2%), and there were no other complications seen such as perforation, bleeding, or sepsis.

More recently, Varadarajulu *et al*^[46] performed a randomized, prospective trial comparing pull-type and

needle-knife EPS in pancreatic-type SOD patients^[47]. The aim of the study was to assess the relative safety of each method of sphincterotomy. Consecutive patients who were diagnosed with pancreatic SOD by manometry were randomized to receive EPS by pull-type or needle-knife sphincterotomy. The primary outcome was the rate of post-ERCP pancreatitis, and secondary outcomes included the rate of endoscopic re-intervention and the response to therapy. A total of 48 patients were enrolled, with 24 in each group. Seven patients (29%) in the pull-type EPS group developed pancreatitis, as compared to none in the needle-knife group ($P = 0.01$). Three patients (12.5%) in the pull-type group required a re-intervention of some kind, versus two (8.3%) in the needle-knife group. Response to endoscopic therapy was the same in each group. The authors concluded that EPS in high-risk patients such as those with SOD is safer if performed with a needle-knife over a pancreatic stent.

Attwell recently compared the complication rates of minor papillotomy using either pull-type or needle-knife technique in 184 patients with pancreas divisum^[48]. In this single-center study, there was no significant difference in the rates of post-ERCP pancreatitis or post-papillotomy bleeding. The overall complication rates in each group were 8.3% (needle-knife group) versus 7.8% (pull-type group). However, the authors did conclude that younger age (< 40 years old) was independently associated with higher rates of restenosis and endoscopic reintervention.

Overall, the rate of pancreatitis following a pancreatic sphincterotomy appears to be approximately 10%-12%^[47-51], with a total early complication rate between 10%-15%^[52-56]. Pancreatitis occurs more frequently in those patients with pancreatic-type SOD, rather than those who have it performed for problems associated with chronic pancreatitis^[57-61]. Thorough data concerning the use of pancreatic stents in the prevention pancreatitis following a pull-type sphincterotomy is somewhat lacking^[62-66]. Sherman *et al*^[67] showed that a pancreatic stent used with needle-knife sphincterotomy may limit the frequency of post-procedural pancreatitis in SOD patients. The problem, however, is that if the stent is left in place for too long, it may begin to induce unwanted ductal and parenchymal changes itself. Also, depending on the type of stent used, patients may need to undergo an additional procedure to have this endoprosthesis removed.

Pancreatitis is the most concerning potential complication for those endoscopists who perform EPS. This is mainly because it appears to be the complication over which we have the least amount of control, and also because its effect may be very severe and sometimes lethal^[68]. The decision to place a stent following any sphincterotomy is made on a case-by-case basis. Factors weighed in the decision include the perceived risk of early pancreatitis versus the potential for late complications and the need for an additional procedure.

CONCLUSION

Pancreatic sphincterotomy is an endoscopic technique used for various pancreas and pancreas-related diseases. The current standard of practice utilizes two

different techniques for performing EPS: a pull-type sphincterotomy without prior stent placement, and a needle-knife sphincterotomy over a stent. Historically, the indications for EPS have been less well-defined than those of endoscopic biliary sphincterotomy. Nonetheless, there are at least a few conditions such as chronic pancreatitis and pancreatic SOD in which pancreatic sphincterotomy plays a definite role. The complications associated with EPS are many, although acute pancreatitis appears to be the most common and the most serious of the early complications. Papillary stenosis is a significant late complication of this procedure as well. The technique of pancreatic sphincterotomy continues to be reserved for those endoscopists who perform a relatively high-volume of therapeutic pancreaticobiliary ERCP.

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S- Editor Ma N L- Editor Kremer M E- Editor Li JL



VIRAL HEPATITIS

Antiviral efficacy of adefovir dipivoxil versus lamivudine in patients with chronic hepatitis B sequentially treated with lamivudine and adefovir due to lamivudine resistance

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Received: 2006-12-20 Accepted: 2007-01-01

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Key words: Chronic hepatitis B; Lamivudine; Adefovir; Treatment efficacy

Seo YS, Kim JH, Yeon JE, Park JJ, Kim JS, Byun KS, Bak YT, Lee CH. Antiviral efficacy of adefovir dipivoxil versus lamivudine in patients with chronic hepatitis B sequentially treated with lamivudine and adefovir due to lamivudine resistance. *World J Gastroenterol* 2007; 13(30): 4072-4079

<http://www.wjgnet.com/1007-9327/13/4072.asp>

Abstract

AIM: To compare the antiviral efficacy of adefovir (ADV) in lamivudine (LMV)-resistant patients with LMV treatment in nucleoside-naïve patients, using serum samples collected sequentially during the course of treatment progressing from LMV to ADV.

METHODS: Forty-four patients with chronic hepatitis B (CHB) were included. The patients were initially treated with LMV and then switched to ADV when LMV resistance developed. Antiviral efficacy was assessed by measuring the following: reduction in serum HBV DNA from baseline, HBV DNA negative conversion (defined as HBV DNA being undetectable by the hybridization assay), and HBV DNA response (either HBV DNA level $\leq 10^5$ copies/mL or a $\geq 2 \log_{10}$ reduction from baseline HBV DNA level).

RESULTS: After two and six months of treatment, HBV DNA reduction was greater with LMV compared to ADV treatment ($P = 0.021$). HBV DNA negative conversion rates were 64% and 27% after one month of LMV and ADV treatment respectively ($P = 0.001$). Similarly, HBV DNA response rates were 74% and 51% after two months of LMV and ADV treatment respectively ($P = 0.026$). The time taken to HBV DNA negative conversion and to HBV DNA response were both delayed in ADV treatment compared with LMV.

CONCLUSION: The antiviral efficacy of ADV in LMV-resistant patients is slower and less potent than that with LMV in nucleoside-naïve patients during the early course of treatment.

INTRODUCTION

Chronic hepatitis B (CHB) is a common disease, with an estimated prevalence of approximately 5% of the world's population^[1]. Carriers of hepatitis B virus (HBV) are at an increased risk of developing cirrhosis, hepatic decompensation, and hepatocellular carcinoma^[2], and these complications result in greater than 1 million deaths annually^[3]. Therefore, the ultimate goal of therapy is to limit or reverse the progression of the disease by sustained suppression of HBV replication^[4]. This goal can be achieved with the use of well-tolerated antiviral agents that provide clinical benefit without inducing resistance. Lamivudine (LMV) and adefovir dipivoxil (ADV) are safe and efficacious drugs licensed for the treatment of CHB. LMV was the first oral drug licensed for the treatment of CHB. It increases hepatitis B e antigen (HBeAg) seroconversion, improves HBV-associated liver disease and reduces the progression of hepatic fibrosis, and the development of cirrhosis^[5-8]. However, selective amplification of LMV-resistant mutants is the main concern with long-term LMV treatment^[9,10]. The prevalence of resistant mutants is 16%-32% during the first year of treatment and increases by approximately 15% with each year of additional treatment^[11-14]. Emergence of LMV-resistance has been reported to be associated with diminished clinical and virological response to LMV^[6,15,16]. Exacerbation of CHB was reported to develop in 40.6% patients carrying LMV-resistant mutants during continued LMV treatment^[17]. LMV-resistance is associated with advanced hepatic fibrosis and severe microinflammatory changes in patients with recurrent

HBV infection after liver transplantation^[18]. Furthermore, hepatic decompensation and death can occur, particularly in patients with cirrhosis^[17,19-22]. In addition, the risk of hepatocellular carcinoma may be increased in patients with LMV-resistance^[23]. Therefore, management of LMV-resistant mutants is a major concern in clinical practice.

ADV use is associated with a low incidence of viral resistance^[24-26] and this drug has potent antiviral efficacy in nucleoside-naïve patients with CHB, resulting in significant biochemical, virological, and histological improvement. Moreover, ADV is efficacious against LMV-resistant HBV^[27-29]. With an increasing number of patients undergoing prolonged LMV treatment, the potential candidates for ADV is growing. However, there are no reports on a direct comparison between the use of ADV for treating patients with LMV-resistant hepatitis B, and the use of LMV for treating nucleoside-naïve patients, in terms of antiviral efficacy and the duration of treatment required.

The present study was carried out to compare the antiviral efficacy of ADV in patients with LMV-resistant strains and LMV in nucleoside-naïve states, using serum samples collected sequentially from 44 patients with CHB during the course of progression from LMV to ADV treatment.

MATERIALS AND METHODS

Materials

Data was collected retrospectively from 44 patients treated with LMV initially and switched to ADV because of development of LMV-resistant HBV infection. Serum samples were obtained at baseline and 1, 2, 3, 6, and 12 mo after commencement of LMV and ADV treatment and kept at -70°C until HBV DNA levels were measured by real time PCR. All patients were negative for antibodies to human immunodeficiency virus and hepatitis C.

Tests for biochemical liver-functions and viral replication, including HBeAg, anti-HBe antibodies and HBV DNA levels, were assessed every 1-3 mo during the treatment period. The HBV DNA levels were quantified using both the hybridization technique (HBV Test, Hybrid Capture II, Digene Corp., Gaithersburg, MD; detection limit, 0.5 pg/mL) and real time PCR assay (GeneMatrix Inc, Seoul, Korea; detection range, $366-3.66 \times 10^{11}$ copies/mL).

The protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by Korea University Guro Hospital human research committee.

Definitions of antiviral treatment response

Antiviral treatment efficacy was determined by the reduction in HBV DNA levels from baseline, HBeAg seroconversion, HBV DNA negative conversion, and HBV DNA response. HBeAg seroconversion was defined as the loss of HBeAg and detection of anti-HBe antibodies in patients whose baseline HBeAg was positive. HBV DNA negative conversion was defined as loss of HBV DNA determined by the hybridization assay on two or more consecutive occasions, at least three months

apart. HBV DNA response was defined as HBV DNA level $\leq 10^5$ copies/mL or a $\geq 2 \log_{10}$ reduction from baseline HBV DNA level^[33]. Viral breakthrough (V-BT) was defined as the reappearance of HBV DNA, measured by the hybridization assay, in patients whose level had become undetectable for at least three months after commencement of antiviral treatment.

HBV DNA quantification by real time PCR

Quantitative analysis of serum HBV DNA was performed retrospectively from stored serum samples. Viral DNA was extracted using Qiagen Blood Kits (Qiagen, Chatworth, CA, USA) according to the manufacturer's instructions. PCR amplifications were performed using a 25 μ L reaction mix containing 300 nmol/L of the forward and 900 nmol/L reverse primers, and 250 nmol/L TaqMan probe (Perkin Elmer Biosystems, Foster City, CA, USA), TaqMan universal PCR masterMix (Applied Biosystems, Foster City, CA, USA) and 5 μ L HBV DNA. An ABI prism model 7300 (Applied Biosystems) continuously detected amplified signals. The following real time PCR amplification protocol was used: (1) initial minimal re-amplification of carry over product with uracil-N-glycosylase (AmpErase, Applied Biosystems) at 50°C for 10 min, and (2) a double round of amplification and quantification involving: 45 cycles at 95°C for 15 s and at 60°C for 60 s. The respective sequences of forward primer, reverse primer and TaqMan probe were as follows: 5'-CCgTCTgTgCCTTCTCATCTg-3' (HBV1F, nucleotides 1549-1569), 5'-AgTCCAAGgTTCTCTTATgYAAgACCTT-3' (HBV1R, nucleotides 1641-1669), and 5'-FAM-CCgTgTgCACTTCgCTTCACCTCTgC-TAMRA 3' (HBV1TAQ, nucleotides 1575-1600). Nucleotide sequence positions were numbered according to Ono *et al.*^[31].

The absolute amount of HBV DNA was quantified using a standard curve generated from subcloned pUC119 (Takara, Japan), a recombinant plasmid containing the entire 3.2 kb of HBV DNA. The linear dynamic range of detection was $366-3.66 \times 10^{11}$ copies/mL.

LMV-resistant mutation analysis

Aliquots of 2 μ L of viral DNA were used for PCR reactions. For genotyping, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS; Bruker Daltonics Biflex IV, Billerica, MA, USA), termed RFMP, PCR was performed in 18 μ L of reaction mixture containing 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 0.2 mmol/L of each dNTP, 10 pmol of each primer, and 0.4 units of Platinum[®] Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The amplification conditions included initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The sequences of forward and reverse primers used in the PCR were respectively: 5'-TTCCCCCACTgTTTggCTggATgTCAGTTAT-3' (nucleotide numbers 712-738) and 5'-TACAgACTTggCCCCCAATACCACATgA-3' (nucleotide numbers 771-744). To insert a new FokI digestion site or to eliminate the naturally occurring FokI site in the products, sequences underlined in each primer

Table 1 Baseline characteristics of 44 patients with chronic hepatitis B sequentially treated with lamivudine and adefovir dipivoxil

	Lamivudine	Adefovir	P
ALT level (IU/L)	310 ± 251	336 ± 379	NS
Total bilirubin level (mg/dL)	1.7 ± 2.1	1.5 ± 1.0	NS
Albumin level (g/dL)	3.8 ± 0.6	3.9 ± 0.7	NS
Positive for HBeAg [Nos. (%)]	39 (88.6)	33 (75.0)	NS
HBV DNA level (log copies/mL) ¹	7.64 ± 0.77	7.36 ± 1.16	NS

Values are expressed as mean ± SD. ¹Serum HBV DNA levels were measured by real time PCR (with a lower limit of 366 copies/mL) and log-transformed with the use of a base-10 scale. ALT: alanine aminotransferase; HBeAg: hepatitis B e antigen; HBV: hepatitis B virus; Adefovir: adefovir dipivoxil; NS: not significant.

were modified as described in Hong *et al*^[32]. PCR was performed as above to amplify the HBV polymerase gene encoding the YMDD motif for cloning or for sequencing analyses. Nucleotide sequence positions were numbered according to Ono *et al*^[31].

Restriction enzyme digestion of PCR products was performed by mixing the PCR reaction mixture with 10 µL of buffer containing 50 mmol/L potassium acetate, 20 mmol/L Tris-acetate, 10 mmol/L magnesium acetate, 1 mmol/L dithiothreitol and 1 unit of FokI. The reaction mixture was incubated at 37°C for 2 h and further incubated at 45°C for 2 h with BstF5I. The resulting digest was desalted by vacuum filtration through a 384-well sample preparation plate containing 5 mg of polymeric solvent (Waters, Milford, MA, USA) per well. The desalted reaction mixtures were resuspended with matrix solution containing 50 mg/mL 3-hydroxy picolinic acid, 0.05 mol/L ammonium citrate and 30% acetonitrile, and were spotted in 3 µL volumes on a polished anchochip plate. Mass spectra were acquired on linear Bruker Daltonics MALDI-TOF MS workstation in a positive ion, delayed extraction mode.

Statistical analysis

The data was analyzed using the statistical package SPSS (version 10.0; SPSS Inc., Chicago, IL, USA). The results are expressed as the means ± standard deviations (SD). HBV DNA levels are expressed as logarithmic scales. Quantitative values are expressed as means and ranges, and were compared using the Student's *t*-test or the Mann-Whitney nonparametric *U* test. Kaplan-Meier estimates and log-rank analyses were used to identify factors associated with the time to HBeAg seroconversion. Qualitative values were correlated with Chi-square or Fisher exact tests. In all cases, *P* < 0.05 (two-tailed) was considered statistically significant.

RESULTS

Baseline characteristics

Table 1 summarizes the baseline characteristics of 44 patients with CHB sequentially treated with LMV and ADV. The mean age was 45 ± 11.2 years (range, 17-67). Thirty patients were men and 13 had cirrhosis. LMV treatment was given for a mean of 29 ± 15.4 mo (range, 7-68). During

Table 2 Types of lamivudine (LMV)-resistant mutations in 40¹ patients in whom analysis of LMV-resistant mutation was performed

Types of LMV-resistant mutations	Patients <i>n</i> (%)	HBV DNA level ²
M204I	8 (20.0)	7.5 ± 0.7
M204V	1 (2.5)	8.0 ³
M204I + L180M	19 (47.5)	7.5 ± 0.9
M204V + L180M	12 (30.0)	7.8 ± 0.5

¹In the remaining four patients, LMV-resistant mutations were diagnosed clinically by the reappearance of hepatitis B virus (HBV) DNA after HBV DNA negativization (determined by hybridization assay). ²Serum HBV DNA levels were measured by real time polymerase chain reaction (PCR), with a lower limit of 366 copies/mL and subjected to log₁₀ transformation. ³HBV DNA level of mutation M204V was expressed only as a mean value because *n* = 1. LMV: lamivudine; HBV: hepatitis B virus.

LMV treatment, cirrhosis developed in five additional patients. V-BT emerged at a mean of 17 ± 8.5 mo of LMV treatment (range, 5-44 mo).

Because of the development of LMV-resistant HBV, all patients were treated with ADV. In 17 patients, LMV was maintained for the initial 1-4 mo (median, 3 mo) of ADV treatment and in 17 patients, LMV was discontinued with the commencement of ADV treatment. The remaining 10 patients were treated with ADV after a treatment-free period of 5 mo (median, range, 1-15 mo).

The baseline serum alanine aminotransferase (ALT), total bilirubin and albumin levels and HBeAg status were not significantly different between the LMV and ADV treatment groups. The mean baseline HBV DNA levels were 7.64 and 7.36 log₁₀ copies/mL respectively.

The different types of LMV-resistance mutations are summarized in Table 2. This analysis was performed in 40 of the 44 study patients; in remaining four, LMV-resistance was diagnosed clinically by the reappearance of HBV DNA (assessed by hybridization assay) after initial HBV DNA negative conversion. The most common LMV-resistance mutation was M204I with L180M (48% of patients). M204V with L180M developed in 30% patients. The M204I and M204V mutations developed in 20% and 3% patients, respectively. Baseline HBV DNA levels did not differ between the four types of LMV-resistant mutations, as shown in Table 2.

Reduction of HBV DNA levels from treatment baseline

The decline in serum HBV DNA from the baseline level after LMV or ADV treatment was measured using real time PCR and the results are shown in Figure 1 as log₁₀ copies/mL (mean ± SD). After one month of antiviral treatment, the mean reduction in HBV DNA levels was 2.3 and 1.8 during LMV and ADV treatment respectively (*P* = 0.121). However, after two months of treatment, LMV treatment produced a significantly greater decline in the serum HBV DNA level compared to ADV (2.7 ± 1.2 *vs* 2.1 ± 1.2; *P* = 0.021). Furthermore, after six months of treatment, HBV DNA levels fell by 3.2 ± 1.4 with LMV treatment compared to 2.5 ± 1.6 with ADV treatment (*P* = 0.030).

At treatment month 12, HBV DNA reduction from baseline in the LMV and ADV treatment groups was 2.3

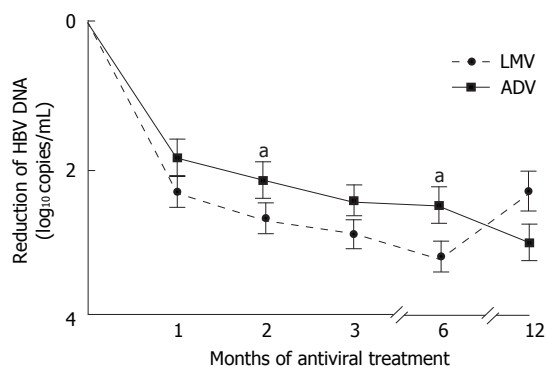


Figure 1 Mean log₁₀ changes in serum hepatitis B virus (HBV) DNA levels after administration of lamivudine (dashed line) and adefovir (continuous line). Mean (\pm SD) changes from baseline in serum HBV DNA concentrations were evaluated using real time PCR assays (lower limit of detection, 366 copies/mL). ^a $P < 0.05$. HBV: hepatitis B virus; LMV: lamivudine; ADV: adefovir dipivoxil.

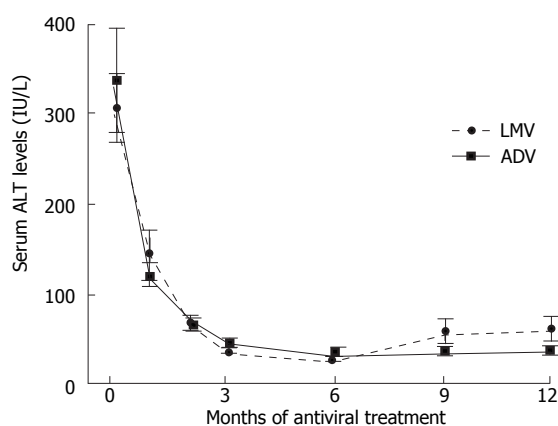


Figure 2 Mean changes in serum alanine aminotransferase levels after administration of lamivudine (dashed line) and adefovir (continuous line). The levels were obtained at baseline and after 3, 6, 9 and 12 mo of lamivudine and adefovir treatment. The values are not significantly different ($P > 0.05$). ALT: alanine aminotransferase; LMV: lamivudine; ADV: adefovir dipivoxil.

and 3.0, respectively ($P = 0.181$). During each 12 mo of treatment with LMV and then ADV, 14 (32%) patients and two (5%) patients developed V-BT, respectively.

When ADV treatment was started, LMV therapy was continued for the first 1–3 mo in 17 patients. The reduction in HBV DNA from the baseline level during ADV treatment was not different between those receiving LMV/ADV combination therapy compared to those receiving ADV alone. HBV DNA reduction from baseline level was not different in the four types of LMV-resistant mutations.

Biochemical response

Figure 2 shows the changes in serum ALT levels before and during antiviral treatment. The mean serum ALT levels during 12 mo of antiviral treatment were not different between LMV and ADV treatment regimens. Serum ALT levels normalized in 40 (91%) patients during 12 mo of LMV treatment, and in 39 (86%) patients during 12 mo of ADV treatment ($P = 0.551$). The time taken to ALT normalization was 4.0 ± 3.55 mo and 5.3 ± 5.18 mo

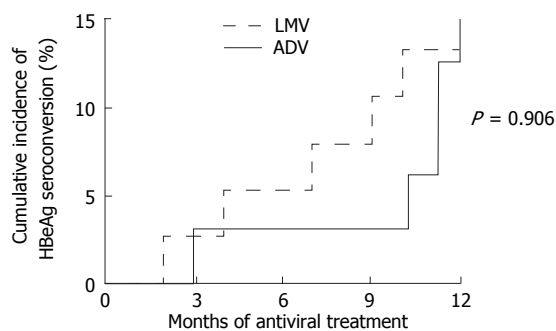


Figure 3 Cumulative rates of sustained hepatitis B e antigen (HBeAg) seroconversion during one year of treatment with lamivudine (dashed line) and after switching to adefovir (continuous line). Only patients with positive HBeAg values at baseline were included in the analysis. Cumulative rates after one-year treatment of lamivudine and adefovir were 13% and 15%, respectively. LMV: lamivudine; ADV: adefovir dipivoxil; HBeAg: hepatitis B e antigen.

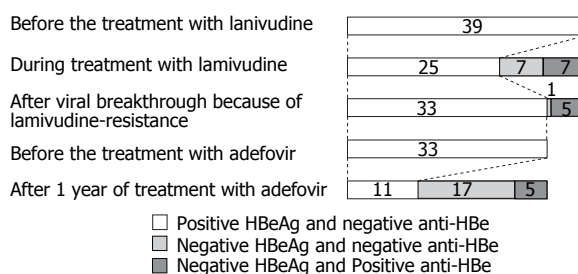


Figure 4 Changes in the status of hepatitis B e antigen (HBeAg) and anti-HBe after treatment with lamivudine and adefovir in patients with positive HBeAg. Before treatment with lamivudine, HBeAg was positive in 39 patients. After one year of treatment, 7 patients became HBeAg negative and 2 of these became anti-HBe positive. During treatment with lamivudine, 14 patients became HBeAg negative and 7 of these became anti-HBe positive. After development of viral breakthrough because of lamivudine-resistance, HBeAg reappeared in 8 of the 14 patients. HBeAg was positive in 33 patients before the treatment with adefovir. HBeAg became negative in 22 patients and 5 of them became anti-HBe positive after one year of treatment. The numerals in the boxes represent the number of patients according to the status of HBeAg and anti-HBe.

after the commencements of LMV and ADV treatment respectively ($P = 0.081$).

HBeAg seroconversion

The cumulative rates of sustained HBeAg seroconversion during one year of antiviral treatment were not different between LMV and ADV (Figure 3). Cumulative rates after one year of treatment with LMV and ADV were 13% (5 of 39 patients) and 15% (5 of 33 patients), respectively. Two (40%) of the five patients whose HBeAg had seroconverted during one year of LMV treatment, reconverted to HBeAg positive status during V-BT. The changes in the HBeAg and anti-HBe status before and during treatment with LMV are shown in Figure 4.

HBV DNA negative conversion

HBV DNA negative conversion (based on hybridization assay) was found in 27% patients after one month of ADV treatment, compared with 64% of patients after one month of LMV therapy (Figure 5A; $P = 0.001$). The proportion of patients who achieved HBV DNA

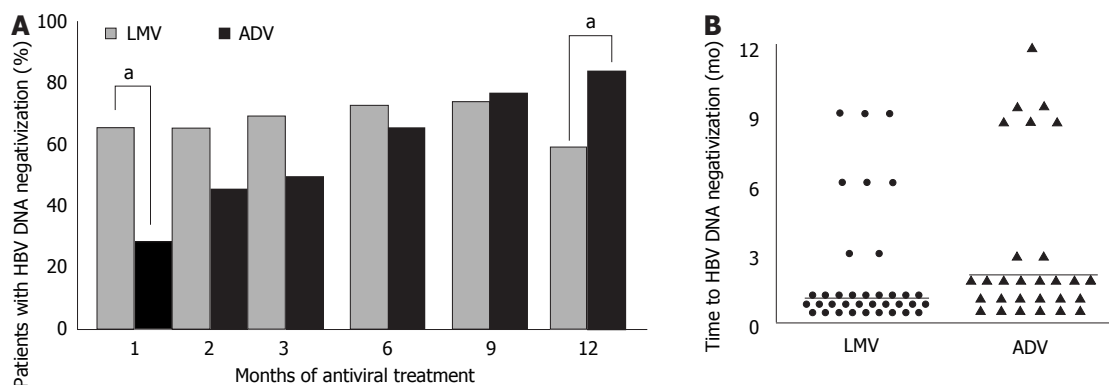


Figure 5 A: Percentage of patients with undetectable HBV DNA (by hybridization assay) at months 1, 2, 3, 6, 9, and 12 after treatment with lamivudine (gray bars) and after switching to adefovir (black bars). $^aP < 0.05$ vs ADV; **B:** Time to HBV DNA loss during 12 mo of lamivudine (left) and adefovir (right) treatment. HBV DNA became negative in 36 patients with lamivudine and in 28 patients with adefovir. Note that HBV DNA negativization took about 1 mo longer with adefovir (median 2 mo) compared to lamivudine (median 1 mo; $P < 0.05$). Times to HBV DNA negativization (by hybridization assay) after lamivudine or adefovir treatment in each patient is represented as ● and ▲, respectively. Horizontal bars (—) represent median time to HBV DNA negativization. LMV: lamivudine; ADV: adefovir dipivoxil.

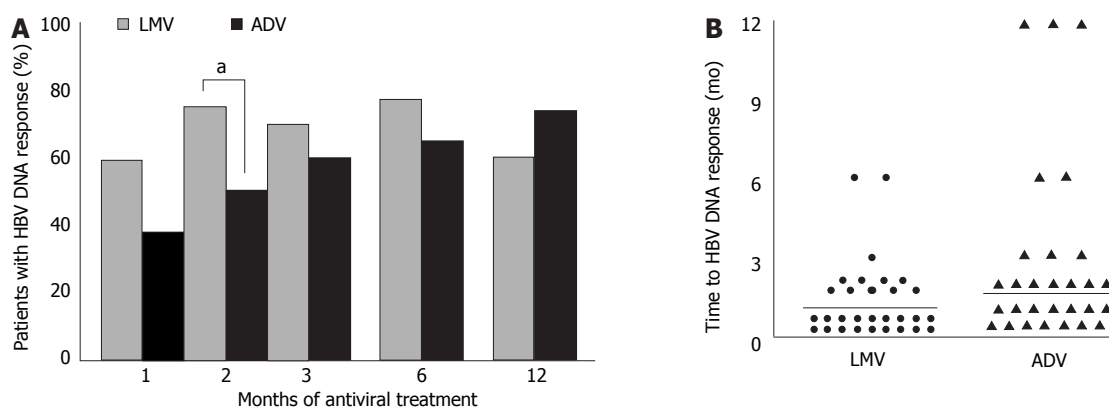


Figure 6 A: Percentage of patients with HBV DNA responses at months 1, 2, 3, 6, 9, and 12 after treatment with lamivudine (gray bars) and adefovir (black bars). $^aP < 0.05$ vs ADV; **B:** Time to HBV DNA response during 12 mo of lamivudine (left) and adefovir (right) treatments. HBV DNA responses appeared in 36 patients with lamivudine treatment and in 30 patients with adefovir treatment. HBV DNA response was slower when patients were treated with adefovir (median 1.5 mo) than with lamivudine (median 1 mo; $P < 0.05$). Time to HBV DNA response after lamivudine or adefovir treatment in each patient is shown as ● and ▲, respectively. Horizontal bars (—) represent median times to HBV DNA response. LMV: lamivudine; ADV: adefovir dipivoxil.

negative conversion increased with the duration of ADV treatment to 83% after 12 mo of treatment. By contrast, HBV DNA negative conversion decreased to 59% after 12 mo of LMV treatment, because of the development of V-BT.

The mean time to HBV DNA negative conversion from the beginning of antiviral treatment was longer in the ADV treatment group (3.5 ± 2.9 mo; median 2 mo) compared to the LMV treatment group (2.0 ± 2.1 mo; median 1 mo) (Figure 5B; $P = 0.020$).

HBV DNA response

HBV DNA response was seen in 59% and 38% patients after one month of LMV and ADV treatment respectively ($P = 0.46$). After two months of treatment, HBV DNA response was seen in 51% patients on ADV compared to 74% on LMV treatment (Figure 6A; $P = 0.026$). The HBV DNA response rate increased to 73% after 12 mo of treatment with ADV. By contrast, HBV DNA response decreased to 61% after 12 mo of LMV treatment, because of the development of V-BT.

The mean time to HBV DNA response from the beginning of antiviral treatment was longer with ADV treatment (2.9 ± 3.4 mo; median 1.5 mo) than LMV treatment (1.6 ± 1.2 mo; median 1 mo; Figure 6B, $P = 0.001$).

DISCUSSION

The antiviral efficacy of ADV has been reported to be similar to that of LMV in nucleoside naïve patients with CHB^[25,26,33,34]. Moreover, a recent *in vitro* study showed that LMV-resistant mutants remained sensitive to ADV^[27]. These findings have been supported by several clinical studies, which showed similar antiviral efficacy of ADV against wild type HBV and LMV resistant HBV^[28,29,35-37]. In these studies, HBV DNA reduction after ADV treatment in nucleoside-naïve patients and LMV-resistant patients were 2.9-3.9 and 2.5-4.3 log₁₀ copies/mL, respectively. However, the antiviral efficacy of ADV in patients with LMV-resistance appears to be slower and less potent compared with the response to LMV in nucleoside-naïve patients; although the evidence in support of this

observation is limited. The present study was designed to provide an answer to this question by direct comparison of antiviral efficacy of LMV and ADV using serum samples collected sequentially from patients with CHB who had earlier received LMV, and who had been switched to ADV because of the appearance of LMV resistance.

In our study, HBV DNA levels were quantified by two methods; hybridization assay and real time PCR. Real-time PCR has a very high sensitivity, although it has not been widely used clinically until recently. At the time our patients were under treatment with LMV, real time PCR was an experimental technique and most clinicians were using the hybridization assay to quantify HBV DNA. Therefore, the majority of studies have used such data to compare the antiviral efficacy of ADV in LMV-resistant patients with that of LMV in nucleoside-naïve patients. To assess the antiviral efficacy of LMV and ADV, it therefore seemed necessary to analyze the data obtained by the hybridization assay.

In our study, the suppression of serum HBV DNA levels after two and six months of treatment was lower with ADV compared to LMV. This finding is in contrast with a recent report that indicated that LMV-resistant mutations resulted in increased van der Waals contacts between ADV and the mutated residues, accounting for the superior binding affinity of ADV with these mutants^[38]. Recently, Ono *et al*^[39] reported that the median effective concentration values of ADV for LMV-resistant mutants were 4-16 times higher than those for wild-type HBV, and suggested that higher doses of ADV will be required for the treatment of LMV-resistant mutants. Our results support this suggestion.

To assess the antiviral efficacy of LMV and ADV, we compared the proportion of patients with HBV DNA negative conversion, and the time required to achieve this after LMV and ADV treatment. After one month of treatment, the conversion rate was significantly lower with ADV compared to LMV treatment (27% *vs* 64%). In addition, HBV DNA negative conversion took significantly longer after ADV than LMV. We also analyzed the proportion of patients with HBV DNA responses, and the time taken after LMV and ADV treatments. An HBV DNA response was defined as an HBV DNA level $\leq 10^5$ copies/mL or a $\geq 2 \log_{10}$ reduction from the baseline HBV DNA level, according to the criteria proposed by Perrillo *et al*^[30]. In their study, HBV DNA response occurred in 85% of LMV-resistant patients after one year of treatment with ADV. Similarly, Locarnini *et al*^[40] defined the antiviral response as $\geq 1 \log_{10}$ reduction in HBV DNA from the baseline level within three months of treatment. In our study, HBV DNA response rate after two months of treatment was significantly lower with ADV compared to LMV (51% *vs* 74%). The HBV DNA response was also significantly delayed after ADV treatment compared with LMV.

In our study, 17 of the 44 patients treated with ADV also received LMV for the initial 1-4 mo of ADV treatment. Because such a combination could influence the overall antiviral efficacy, we compared the efficacy of HBV DNA suppression between patients who received combination treatment and those who did not, and found no significant difference in the results (data not shown).

Although the antiviral efficacy of LMV was faster than that with ADV during the first several months of treatment, the development of V-BT reduced this advantage of LMV after 12 mo of treatment. By contrast, the antiviral efficacy of ADV increased with time, with a low incidence of ADV-resistance. Therefore, ADV appears to be superior to LMV under conditions that require long-term antiviral treatment. When we compared the rate of HBV DNA reduction from the baseline level after the exclusion of patients who developed V-BT within 12 mo of antiviral treatment, there was no significant difference between LMV and ADV treatments.

The probability of a mutant strain being selected during therapy depends upon the ability of a drug to suppress viral replication^[41]. Using a more potent antiviral drug during the initial course of treatment may reduce the chances of selection of drug resistant mutants^[40,41]. Therefore, it is necessary to determine whether using ADV rather than LMV for the initial treatment might affect the incidence of drug resistance in long-term nucleoside/nucleotide-treated patients. In our study, only two of 44 (5%) patients developed V-BT after 12 mo of treatment because of ADV-resistant mutation, which was significantly lower than the incidence of V-BT caused by LMV-resistant mutations (32%, $P = 0.002$). However, the incidence of V-BT caused by ADV-resistant mutations was higher than that reported previously^[24]. Our findings are consistent with a recent study which showed that the emergence of the ADV mutations in LMV-resistant patients appeared to occur earlier and was more frequent than in nucleoside-naïve patients^[42]. Further studies are needed to determine whether the less potent and slower antiviral efficacy of ADV in the early treatment course in LMV-resistant patients could lead to a higher incidence of ADV-resistant mutations.

The main limitation of our study was that we compared the efficacy of ADV in LMV-resistant patients with that of LMV in nucleoside-naïve patients and not with that of ADV in nucleoside-naïve patients. It should be noted that we compared the antiviral efficacy of two different drugs, nucleoside analogue (LMV) and nucleotide analogue (ADV) under different conditions: a nucleoside-naïve state and an LMV-resistant state. However, our study was designed to analyze any difference in the antiviral efficacy, and the time taken to achieve sufficient HBV DNA suppression after commencement of antiviral therapy when LMV was switched to ADV because LMV resistance had developed. We demonstrated that ADV had slower and less potent antiviral effect, which most clinicians have suspected until now using the hybridization assays, and verified these findings using real time PCR.

In conclusion, the antiviral efficacy of ADV in patients with LMV-resistant HBV appears to be slower and less potent than that of LMV against wild type HBV during the early course of treatment. However, the superior initial antiviral efficacy of LMV was reduced in the later course of treatment because of the appearance of drug resistant viral mutations.

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S- Editor Wang J **L- Editor** Anand BS **E- Editor** Liu Y



VIRAL HEPATITIS

Hepatitis C Virus non-structural 5A abrogates signal transducer and activator of transcription-1 nuclear translocation induced by IFN- α through dephosphorylation

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Supported by National Natural Science Foundation of China, No. 39670671, No. 30471531

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Received: 2007-03-06 Accepted: 2007-03-28

protein, which might be another possible resistance mechanism to interferon alpha therapy.

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Key words: Hepatitis C virus nonstructural protein 5A; IFN- α ; Signal transducer and activator of transcription (STAT1); Phosphorylation; Nuclear translocation

Gong GZ, Cao J, Jiang YF, Zhou Y, Liu B. Hepatitis C Virus non-structural 5A abrogates signal transducer and activator of transcription-1 nuclear translocation induced by IFN- α through dephosphorylation. *World J Gastroenterol* 2007; 13(30): 4080-4084

<http://www.wjgnet.com/1007-9327/13/4080.asp>

Abstract

AIM: To study the effect of Hepatitis C virus non-structural 5A (HCV NS5A) on IFN α induced signal transducer and activator of transcription-1 (STAT1) phosphorylation and nuclear translocation.

METHODS: Expression of STAT1 Tyr701 phosphorylation at different time points was confirmed by Western blot, and the time point when p-STAT1 expressed most, was taken as the IFN induction time for further studies. Immunocytochemistry was used to confirm the successful transient transfection of NS5A expression plasmid. Immunofluorescence was performed to observe if there was any difference in IFN α -induced STAT1 phosphorylation and nuclear translocation between HCV NS5A-expressed and non-HCV NS5A-expressed cells. Western blot was used to compare the phosphorylated STAT1 protein of the cells.

RESULTS: Expression of HCV NS5A was found in the cytoplasm of P^{NS5A-transfected} Huh7 cells, but not in the PRC/CMV transfected or non-transfected cells. STAT1 Tyr701 phosphorylation was found strongest in 30 min of IFN induction. STAT1 phosphorylation and nuclear import were much less in the presence of HCV NS5A protein in contrast to P^{RC/CMV-transfected} and non-transfected cells under fluorescent microscopy, which was further confirmed by Western blot.

CONCLUSION: HCV NS5A expression plasmid is successfully transfected into Huh7 cells and HCV NS5A protein is expressed in the cytoplasm of the cells. IFN- α is able to induce STAT1 phosphorylation and nuclear translocation, and this effect is inhibited by HCV NS5A

INTRODUCTION

Hepatitis C virus (HCV) infection has become a major problem of public health with more than 100 million infected individuals worldwide, including over 30 million in China. Unfortunately, up to 60%-80% of HCV infected adults will develop chronic liver disease, although the clinical phenomena may vary greatly from being asymptomatic to liver cirrhosis or hepatocellular carcinoma (HCC)^[1]. Interferon is still the major drug against chronic hepatic HCV infection, but sustained virologic response (SVR) to interferon alone remains low, especially in HCV 1b infected patients. Although a combination of pegylated IFN and ribovirin may greatly improve the SVR and now become the standard therapy, some patients still remain unresponsive. HCV proteins which may be related to interferon resistance include core protein C^[2], envelope protein E^[3], nonstructural protein NS3/4A^[4] and NS5A^[5]. As one of the HCV nonstructural proteins, NS5A plays an important role in the interferon response, viral replication, and hepatic carcinogenesis^[6]. Janus kinase-signal transducer and activator of the transcription (JAK-STAT) signaling pathway is a major interferon-induced signal pathway which executes the anti-virus function. STAT1 is a member of the STAT family, which exists in the cytoplasm as an inactivated monomer. Induced by interferon, STAT1 is Tyr701 phosphorylated and dimerized, then translocated into the nucleus to stimulate ISGs (interferon stimulated genes) transactivation and anti-viral protein expression.

The anti-viral effectiveness of interferon may be inhibited when this STAT pathway is damaged. HCV NS5A protein is supposed to play an important role in HCV resistance to IFN, and one of the mechanisms is to interact with PKR^[5,7,8], but it is still uncertain whether HCVNS5A has any influence on the STAT signal. In this experiment we found that HCV NS5A may inhibit STAT1 Tyr701 phosphorylation and nuclear translocation, which may be a new way of HCV NS5A to interfere with IFN function.

MATERIALS AND METHODS

Cell lines and plasmids transfection

Plasmid P^{RC/CMV} is a gift from Professor Siddiqui (University of Colorado, USA) Plasmids p^{CNS5A} is an eukaryotic expression vector made in Dr. Siddiqui's laboratory, which was constructed by cloning HCV type 1b NS5A cDNA into P^{RC/CMV} plasmid^[9]. The liver carcinoma cell lines Huh7 (preserved at the Institute of Hepatology, The Second Xiangya Hospital, Central South University) were grown in Dulbecco's modified eagle medium (Life Technologies, USA) with 10% fetal bovine serum at 37°C and 5% CO₂. The cells were transfected by the individual plasmids with Lipofectin reagent (Life Technologies, USA) when they became 60%-70% confluent. Forty-eight h after transfection, the cells were harvested for the detection of HCV NS5A protein expression by immunocytochemistry, and the analysis of STAT1 Tyr701 phosphorylation and nuclear translocation by immunocytofluorescence and Western blot.

Western blot experiment

Total cellular proteins of Huh7 cells induced by IFN α -2b (Harbin Pharmaceutical Company, China) at different time points (0.25, 0.5, 1, 2, 4 and 8 h) were extracted as follows: Huh7 cells were collected and 100 μ L lysate solution [150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L NaF, 50 mmol/L Tris-HCl (pH7.4), 1% NP-40, 0.1 mmol/L NaN₃, 50 μ g/mL PMSF] was added. After 30 min incubation on ice and centrifugation at 4°C, 12000 $\times g$ for 10 min, the supernatant was saved as total cellular protein for further studies. The same amount of total protein was used to load onto 10% SDS-PAGE, and then transferred onto a NC membrane (Schleicher & Schuell Company). After blocking in 5% fat-free milk for 1 h, the membrane was probed by anti-phosphorylated STAT1 (Tyr701) (Cell Signaling Technology), washed and incubated with the second HRP-labeled antibody (Boster, China), and then observed by DAB staining (KPL, USA), and anti-actin (Santa Cruz) served as the control.

Immunocytochemistry and immunofluorescence assay

For immunocytochemistry, P^{CNS5A} and P^{RC/CMV}-transfected and non-transfected Huh7, cells were washed three times and fixed with 4% paraformaldehyde, then blocked with 5% normal goat serum. Serum from HCV infected patients as primary antibody was added to bind HCV NS5A protein, followed by HRP-labeled antibody incubation and DAB staining. For immunofluorescent assay, P^{CNS5A}- and P^{RC/CMV}-transfected and non-transfected Huh7

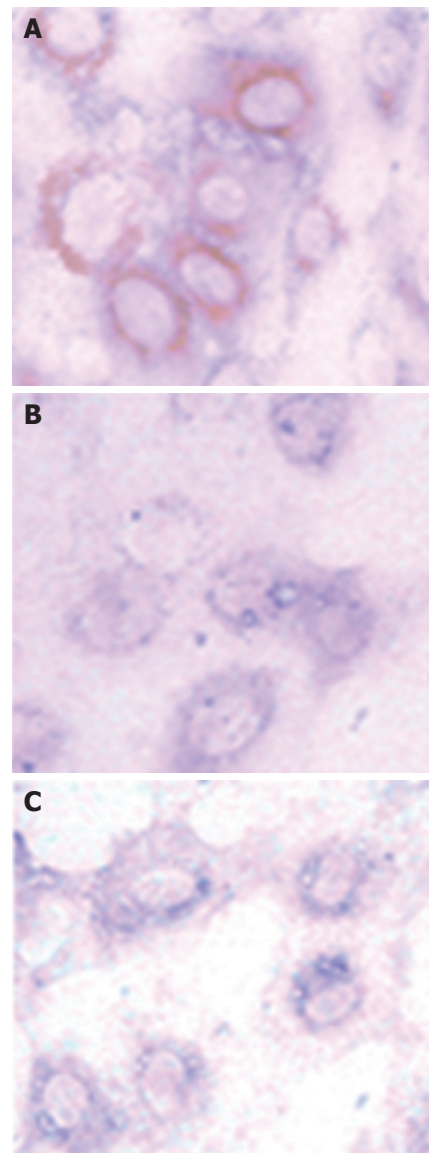


Figure 1 HCV NS5A protein staining of Huh7 cells after 48 h transfection (DAB, $\times 400$). A: P^{CNS5A}-transfected cells; B: P^{RC/CMV}-transfected cells; C: Non-transfected cells.

cells induced by IFN α -2b for 30 min were washed and fixed as described in immunocytochemistry. Anti-STAT1 (Boster, China), anti-phosphorylated STAT1 (Tyr701, Cell Signaling Technology, USA) and FITC-labeled antibody (KPL, USA) were employed, and the staining was observed under fluorescent microscope.

RESULTS

HCV NS5A expression was detected in P^{CNS5A}-transfected cells

HCV NS5A protein was found in brown staining, and distributed in the cytoplasm around the nucleus in some P^{CNS5A}-transfected huh7 cells, but no staining was observed in P^{RC/CMV} transfected or non-transfected cells (Figure 1). This result indicates that NS5A plasmid was successfully transfected into Huh 7 cells and NS5A protein expressed.

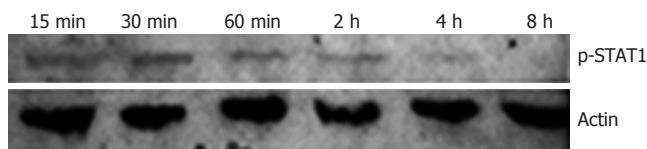


Figure 2 Tyr701 phosphorylated STAT1 in Huh7 cells induced by IFN α -2b (10000 U/mL) (Western blot).

STAT1 Tyr701 phosphorylation after induction of IFN α -2b

STAT1 Tyr701 was mostly phosphorylated after 30-min of induction of IFN α -2b. Western blot showed that the time point of 30 min after IFN α -2b induction was the best for STAT1 Tyr701 phosphorylation, though early to 15 min and later till 4 h after IFN stimulation, the STAT1 phosphorylation was still detected (Figure 2). We therefore chose the 30 min of IFN induction as the time point for further experiments.

Immunofluorescence assay was used to identify the STAT1 phosphorylation and nuclear translocation more clearly. Under fluorescent microscopy, whole STAT1 was found in the plasma of Huh7 cells, but not in nucleus without IFN stimulation. After 30-min induction of IFN α -2b, almost all of the STAT1 translocated into the nuclei (Figure 3A). When anti-phosphorylated STAT1 (Tyr701) was employed as the first antibody, as we anticipated, there was no staining of STAT1 in the cytoplasm of the cells without IFN stimulation, indicating that STAT1 found in the plasma was un-phosphorylated. It was also demonstrated in this experiment that the STAT1 in nucleus was totally stained after 30 min of IFN induction, indicating the STAT1 in the nucleus was phosphorylated (Figure 3B). These results showed that IFN α -2b was able to induce STAT1 phosphorylation and sequential nuclear import.

IFN α -2b-induced STAT1 phosphorylation and nuclear translocation inhibited by HCV NS5A protein

When the JAK-STAT signal pathway was activated by IFN, STAT1 molecules originally distributed in the plasma were phosphorylated at Tyr701 and translocated into the nucleus in a short time. In our experiment, Huh7 cells were induced by IFN α -2b (10000 U/mL) for 30 min. We found that P^{RC/CMV-transfected} and non-transfected cell nuclei were stained for STAT1, and no difference was observed between the two-groups of cells. On the contrary, most P^{CNS5A-transfected} cells were nuclei negative while plasma positive for STAT1, indicating the inhibition of HCV NS5A on IFN-induced STAT1 nuclear import. With regard to the test using anti-phosphorylated STAT1 as the first antibody, most of the NS5A-expressed cell nuclei were not stained for STAT1, suggesting that HCV NS5A inhibited STAT1 phosphorylation. Some cell's nuclei were stained for STAT1, possibly because these cells were not successfully transfected by the NS5A expression plasmid. In the other two groups, all the cell nuclei were positive and plasma was negative for p-STAT1 (Figure 4).

Western blot was performed to further confirm NS5A's inhibition of STAT1 phosphorylation induced by IFN. It turned out that phosphorylated STAT1 (Tyr701) expression was remarkably lower in the presence of HCV

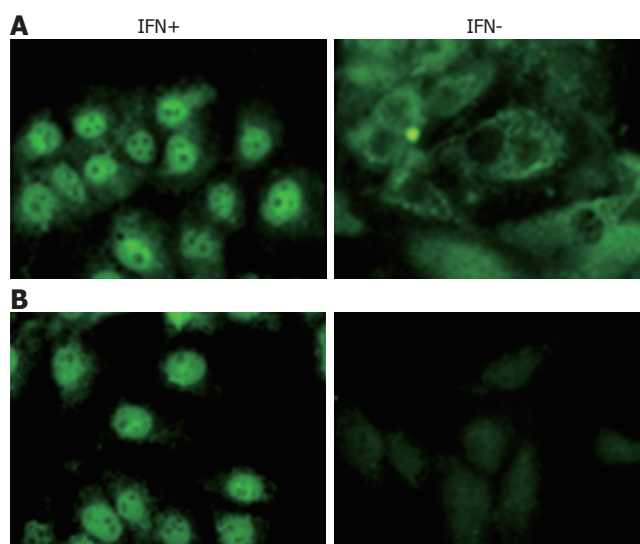


Figure 3 STAT1 or Phosphorylated STAT1 (Tyr701) in Huh7 cells stimulated by IFN α -2b (10000 U/mL) for 30 min (FITC, \times 400). A: STAT1; B: Tyr701.

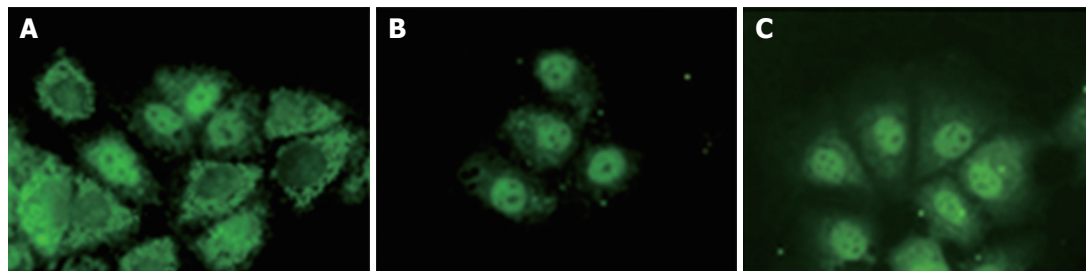
NS5A compared with the other two groups with no NS5A protein expression (Figure 5), indicating that HCV NS5A had an inhibitive effect on STAT1 Tyr701 phosphorylation.

DISCUSSION

HCV is a positive single-stranded RNA virus belonging to the Flaviridae family, and its genome only contains a single long open reading frame encoding a large poly-protein precursor which is thereafter processed by a combination of cellular and viral proteases into several mature proteins, including three or four structural proteins (core, E1, E2/P7) and at least 6 nonstructural proteins (NS): NS2, NS3, NS4A, NS4B, NS5A, NS5B^[10]. HCV NS5A is a phosphoprotein that exists mostly in the cytoplasm in two different forms of Mr 56000 and 58000 with modifications of serine residues. Mr 58000 form is produced by additional phosphorylation of the Mr 56000 form^[11]. In 1995, Enomoto *et al*^[12] first discovered the association between HCV NS5A and sensitivity to interferon therapy. Since then, a lot of work has been done and showed that HCV NS5A might affect an interferon therapeutic effect through several ways, including variation of amino acid in the HCV NS5A ISDR (interferon sensitivity determining region)^[12] or variable region V3^[13,14], interaction between antiviral protein PKR (double-stranded RNA-dependent protein kinase)^[5,7,8] or 2', 5'-OAS (2', 5'-oligoadenylate synthetase)^[15] and others^[16,17]. But it is still uncertain whether HCV NS5A has any effect on the IFN-induced STAT signal pathway. Recently, NS5 protein from other viruses such as Langkat virus (LGTV) and Japanese encephalitis virus have been reported to inhibit IFN-induced STAT signaling^[23,24]. This aroused our interest in knowing if HCV NS5A has similar function.

It is well known that the interferon mediated antiviral effect is mainly through a JAK-STAT pathway, in which STAT1 plays an important role as molecular messenger. STAT1 exists in two isomerides, STAT1 α and splice variant STAT1 β /STAT2. STAT1 α gene knockout rats

STAT1



p-STAT1 (Tyr701)

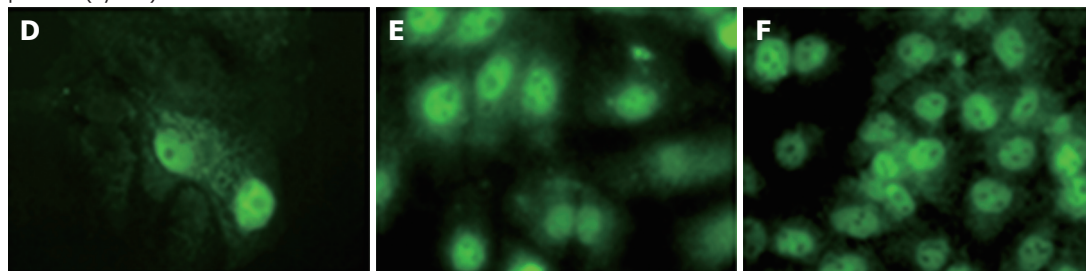


Figure 4 STAT1 or phosphorylated STAT1 in cytoplasm and nuclei of Huh7 cells after 30 min IFN induced (FITC, $\times 400$): **A** and **D**: pCNSA-transfected; **B** and **E**: pRCMV-transfected; **C** and **F**: non-transfected.

shows no response to interferon α or γ ^[18], indicating the criticality of STAT1 in interferon antiviral activity. STAT1 molecules originally distribute in cytoplasm. Induced by interferon, the 701 amino acid residue in the C terminal of STAT1 is phosphorylated and interferon stimulatory gene factors are formed, followed by nuclear import with importin α 5^[19], binding to ISRE (interferon-stimulated response element), and regulation of transactivation and expression of antiviral proteins. So in our experiment, we mainly focused on the possible effect of HCV NS5A on STAT1 phosphorylation and nuclear translocation. The results showed that STAT1 phosphorylation became strong gradually when induced by interferon from 15 to 30 min and decreased quickly in a few hours, which is in accordance with other reports^[19]. When HCV NS5A protein was introduced by transient transfection, IFN-induced STAT1 nuclear translocation was greatly decreased. Since STAT1 Tyr701 phosphorylation is the prerequisite of IFN-induced STAT1 nuclear import, we further proved that STAT1 Tyr701 phosphorylation and sequential nuclear translocation were both reduced in the presence of HCV NS5A, indicating that HCV NS5A interferes with the interferon signaling pathway. It might be one of the HCV NS5A related molecular mechanism in interferon resistance. Interestingly, almost in the same time, Lan also reported the similar result that HCV NS5A protein inhibited IFN-induced STAT1 phosphorylation and nuclear translocation in three hepatocyte-derived cell lines, and found HCV NS5A protein could interact with the N-terminal of STAT1, which would be the molecular mechanism by which HCV NS5A inhibits STAT1 phosphorylation^[25]. They used full-length HCV and HCV subgenomic constructs to transiently transfect Huh-T7 cells. HCV NS5A protein may be affected by other HCV proteins such as NS5B^[26]. Ours and other's results suggested that HCV NS5A protein may inhibit IFN-induced STAT1 phosphorylation and the subsequent nuclear import. One of the mechanisms might be the binding of HCV NS5A and STAT1.

JAK-STAT and MAPK pathway “cross-talk” with

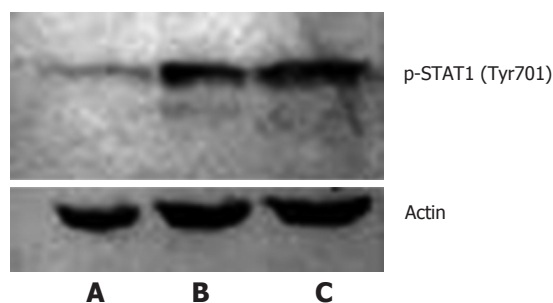


Figure 5 Phosphorylated STAT1 from IFN induced Huh7 cells (FITC, $\times 400$): **A**: pCNSA-transfected; **B**: pRCMV-transfected; **C**: Non-transfected.

each other. Serine phosphorylation can modulate tyrosine phosphorylation, activation and DNA-binding activity of STATs while in the MAPK pathway, activated ERK phosphorylates the conserved Ser727 on STAT3 and the serine phosphorylation enhances tyrosine phosphorylation on Tyr701^[20-22]. Can HCV NS5A interact with the MAPK pathway and indirectly depress STAT1 phosphorylation in the JAK-STAT pathway? Can HCV NS5A disturb the function of importin α 5? These questions need to be addressed in future studies.

ACKNOWLEDGMENTS

We wish to thank Drs. Xian-Shi Su, Xin-Xiang Xiao, Wen-Long Wang and Chun-Ming Deng for their technical help in our study.

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S- Editor Zhu LH L- Editor Ma JY E- Editor Lu W

Baseline HBV DNA level is the most important factor associated with virologic breakthrough in chronic hepatitis B treated with lamivudine

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Supported by the research grant of the Chungbuk National University in 2004

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Received: 2007-05-16 Accepted: 2007-06-04

Abstract

AIM: To identify the factors associated with virologic breakthrough and to select a subgroup of patients who respond well to lamivudine without developing virologic breakthrough (VBT).

METHODS: Of 79 patients who had received lamivudine therapy for 9-57 mo, 34 were HBeAg-positive and 45 were HBeAg-negative, 24 developed virologic breakthrough and 55 did not. Clinical and virologic factors were compared between the two groups.

RESULTS: The median duration of therapy was 25 (9-57) mo. Virologic breakthrough was defined as a > 1 log HBV DNA increase following initial suppression. When several factors, including gender, duration of infection, baseline HBV DNA, and baseline ALT in HBeAg-positive chronic hepatitis patients were analyzed by logistic regression, the most important predictor of virologic breakthrough was the baseline HBV DNA ($r^2 = 0.12$, $P < 0.05$). When HBeAg-positive chronic hepatitis patients were divided into two groups by a point of 6.6 log HBV DNA, the incidence of virologic breakthrough between two groups was significantly different.

CONCLUSION: Lamivudine may remain an effective first line therapy for those HBeAg-positive patients with a baseline HBV DNA < 6.6 log₁₀ copies/mL.

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Key words: Hepatitis B; Lamivudine; Virologic

breakthrough; HBV DNA

Chae HB, Hann HW. Baseline HBV DNA level is the most important factor associated with virologic breakthrough in chronic hepatitis B treated with lamivudine. *World J Gastroenterol* 2007; 13(30): 4085-4090

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INTRODUCTION

A high incidence of virologic breakthrough (VBT) that results from viral resistance is a major disadvantage of lamivudine (LAM) treatment for patients with chronic hepatitis B (CHB). Data from a study of 592 patients who had undergone four years of LAM treatment indicated that resistance increased from 23% in year 1 to 71% in year 4^[1,2]. The clinical consequences of viral resistance can be severe, but early studies suggest that median HBV DNA and ALT levels continue to improve even as resistance develops^[3]. LAM is known to have long-term therapeutic effects, with well-known resistance and side effects. LAM therapy for CHB patients with advanced fibrosis significantly reduced the incidence of hepatic decompensation and hepatocellular carcinoma (HCC)^[4]. In addition, it is becoming increasingly clear that CHB management requires a long-term therapy, thus making the cost of treatment an important consideration. The need for long-term therapy and the consequent financial burden become significant in the economically disadvantaged regions of the world which also happen to be the hyper-endemic areas for HBV. Furthermore, this cost issue presents a serious economic burden for CHB patients who immigrated to the US from the hyper-endemic regions. One study suggested that upfront LAM therapy may be highly cost-effective, assuming that patients who develop resistance begin a regimen of adefovir instead of continuing LAM treatment^[5]. Our study sought to identify the factors associated with VBT in patients on LAM therapy and attempted to select a subgroup of patients who may respond well to LAM without developing VBT.

MATERIALS AND METHODS

Patients

We reviewed the medical records of 208 CHB patients who were treated at the Liver Disease Prevention Center,

Division of Gastroenterology and Hepatology at Thomas Jefferson University Hospital, Philadelphia from January 2000 to December 2004. Ninety-seven patients were excluded because they were treated with other anti-viral agents (excluding interferon) prior to their first visits. Five were excluded due to co-infection with Hepatitis C virus. We selected individuals from the remaining 106 patients who had been on LAM therapy for at least 9 mo (which excluded 14 patients) and whose pre-treatment HBV DNA was greater than $3 \log_{10}$ copies/mL (which excluded 5 patients). Baseline HBeAg status was not available for four patients. Four patients who had suboptimal responses during the entire treatment period were also excluded from the final analysis because of uncertainty of classifying these patients into either the breakthrough or the non-breakthrough group. In the end, clinical and laboratory data from a total of 79 patients were reviewed.

Inclusion and exclusion criteria

The inclusion criteria consisted of the following two conditions: (1) Patients had received LAM therapy for at least 9 mo; and (2) they had a baseline HBV DNA level $\geq 3 \log_{10}$ copies/mL. Patients were excluded if they had previously received anti-HBV therapy (with the exception of interferon therapy), or if they had hepatitis C virus or hepatitis D virus co-infection.

HBsAg, HBeAg, anti-HBe, and HBV DNA quantification

All patients had been positive for hepatitis B surface antigen (HBsAg) for more than 6 mo. HBeAg/anti-HBe and anti-HDV were determined using ELISA (Abbott Laboratories, Chicago, IL), and anti-HCV antibodies were assayed using a third-generation enzyme immunoassay (Abbott Laboratories, North Chicago, IL). Between 2000 and 2002, a solution hybridization assay (Abbott Laboratories, North Chicago, IL) with a lower limit of detection (LLOD) of 1.6 pg/mL was used to measure HBV DNA levels. These values were converted to copies/mL by determining 283 000 copies/mL per 1 pg/mL of HBV DNA. From 2003 until the present, HBV DNA was measured by RT-PCR (Quest Diagnostics, Horsham, PA) with a lower limit of detection of 500 copies/mL. Serial dilutions were performed for samples exceeding $5.3 \log_{10}$ copies/mL. Values below this cutoff were assigned a value of $1 \log_{10}$ copies/mL.

Definitions

The initial virologic response was defined as HBV DNA that was less than $4 \log_{10}$ copies/mL after 6 mo on therapy, and viral suppression was defined as the difference between the level of HBV DNA at baseline and after 6 mo of treatment. Maximal virologic suppression was defined as the difference in HBV DNA levels between baseline and nadir. VBT was defined as $> 1 \log_{10}$ copies increase in HBV DNA from nadir on two consecutive occasions after an initial virologic response or HBV DNA could be detected again after the previous report of "under the detection limit". Suboptimal responders were defined as patients whose initial virologic response was less than 2 log during the entire period of treatment.

Determination of HBV genotype, antiviral resistance, precore mutant, and core promotor mutant

HBV DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The presence of HBV DNA polymerase gene mutations was determined using the InnoLiPA DR2 line probe assay (InnoGenetics, Ghent, Belgium) according to the manufacturer's instructions. Line probe results were confirmed by bi-directional automated sequencing at the DNA sequencing core facility (Thomas Jefferson University's nucleic acid facility, Philadelphia, PA) using the standard protocol for the Applied Biosystems DNA Sequencer 377 (Perkin Elmer Corp., Foster City, CA). This amplicon covers domains A, B, C, D, and E of the reverse transcriptase region of the HBV polymerase. The PCR protocol and primers for the surface/polymerase gene and core promotor/precore region used in this study were described previously^[6].

Statistical analysis

Statistical testing was performed using SPSS version 13.0 (SPSS Inc., Chicago IL). Results were expressed as mean \pm SD or median (minimum-maximum). HBV DNA levels were logarithmically transformed for analysis. Continuous variables were compared using the two-tailed student's *t*-test, and categorical data were compared using the two-tailed χ^2 test. Factors associated with an initial virologic response and LAM resistance were analyzed by univariate analysis. Clinical, biochemical and virologic factors that could influence LAM resistance, including gender, route of infection, baseline ALT level, baseline HBV DNA level, were analyzed by binary logistic regression. The cumulative probability of lamivudine resistance was estimated by Kaplan-Meier analysis. *P* < 0.05 was considered statistically significant.

RESULTS

Baseline characteristics of patients prior to beginning lamivudine treatment

Seventy-nine patients (54 males) met eligibility criteria. All were Asian Americans, with a mean age of 47 ± 12 years. Thirty-seven (45%) had been infected at birth by vertical transmission from their mothers. Twenty-four patients (30%) had liver cirrhosis and two (2%) had liver cancer prior to starting LAM therapy. The mean duration of therapy was 26 ± 10 mo. At baseline, the ALT was 66 (11-3395) IU/L and the mean log HBV DNA level was 6.0 ± 1.6 . The baseline HBV DNA level in the 34 HBeAg-positive patients was 6.6 ± 1.5 , and the baseline DNA level in the 45 HBeAg-negative patients was $5.6 \pm 1.5 \log_{10}$ copies/mL. Treatment was initiated if HBV DNA levels were $\geq 3 \log_{10}$ copies/mL. Eighteen patients had HBV DNA levels between 3 and $5 \log_{10}$ copies/mL. Based on earlier observations of 317 CHB patients^[7], normal pre-therapy ALT was not considered a contraindication for beginning antiviral therapy in certain patients. These included patients who had normal ALT levels, high viral DNA, and a strong family history of HCC or patients who had had previous ALT spikes but whose ALT levels

Table 1 Baseline characteristics of patients (mean \pm SD)

	All patients (<i>n</i> = 79)	Breakthrough (<i>n</i> = 24)	Non-breakthrough (<i>n</i> = 55)
Age (yr)	47 \pm 12	47 \pm 13	47 \pm 11
Female (%)	32	29	33
Family history of CHB (%)	47	46	55
ALT (IU/L)	149 \pm 44	98 \pm 20	171 \pm 63
Total bilirubin (mg/dL)	1.1 \pm 1.1	1.3 \pm 1.4	1.0 \pm 0.9
Albumin (g/dL)	4.4 \pm 0.4	4.2 \pm 0.5	4.3 \pm 0.4
INR	1.07 \pm 0.12	1.09 \pm 0.15	1.06 \pm 0.08
Cirrhosis (%)	30	36	24
HBeAg-positive (%)	43	56	36
HBV DNA log ₁₀ copies/mL ^a	6.0 \pm 1.6	6.6 \pm 1.5	5.7 \pm 1.5
Mean duration of lamivudine treatment (mo)	26 \pm 10	29 \pm 11	25 \pm 10
3 log < HBV DNA < 5 log (%)	19	3	16
HBV DNA \geq 5 log (%)	60	21	39

^a*P* < 0.05 breakthrough vs non-breakthrough group INR: International normalized ratio..

were normal at the beginning of the study. All baseline characteristics except HBV DNA level were similar between the VBT and non-VBT groups. Only ALT level was shown as mean \pm SE (Table 1).

Response to lamivudine treatment

In HBeAg-positive patients (*n* = 34), the mean log reduction (copies/mL) in HBV DNA from baseline was 4.6 \pm 1.4, 3.7 \pm 2.4 and 2.7 \pm 3.0 at 6 mo, 12 mo and 24 mo, respectively. The less reduction in two years may be due to VBT in some patients. As shown in Table 2, initial viral suppression was 4.2 \pm 2.0 and 4.3 \pm 1.5 in VBT and non-VBT groups, respectively. The maximal viral suppression was 4.8 \pm 1.7 and 4.6 \pm 1.2 in VBT and non-VBT groups, respectively. Eighty percent of patients showed an initial anti-viral response at 6 mo post-treatment. HBeAg loss rate was 18, 26, and 29% at 1, 2, and 3 years, respectively. Excluding individuals with normal or absent ALT levels at baseline, ALT normalization was observed in 90% and 96% of patients after 6 mo and 12 mo, respectively. The mean time for VBT detection was 21 (9-36) mo. There was no viral or biochemical variable with a significant difference between VBT and non-VBT groups.

In HBeAg-negative patients (*n* = 45), the mean log reduction (copies/mL) in HBV DNA from baseline was 3.2 \pm 2.0 at 6 mo, 4.2 \pm 1.9 at one year, and 3.1 \pm 3.0 at two years. Initial viral suppression was 2.8 \pm 2.5 and 3.4 \pm 1.9 in VBT and non-VBT groups, respectively. Maximal suppression was 4.3 \pm 1.7 and 4.3 \pm 1.6 in VBT and non-VBT groups, respectively. Eighty-one percent of patients showed an initial antiviral response at 6 mo post-treatment. Excluding individuals with normal or absent ALT levels at baseline, ALT normalization was observed in 84% and 82% of patients after 6 mo and 12 mo, respectively. The mean time for VBT detection was 22 (9-36) mo. Also, there was no viral or biochemical variable with a significant difference between VBT and non-VBT groups.

HBV genotype, precore mutant, and core promotor mutant
HBV DNA sequences were examined in nine patients' sera

that were collected at the time of baseline and VBT. Six patients without VBT were also examined as controls. The samples from 15 patients were investigated for genotype, precore mutant, and core promoter mutant. As shown in Table 3, YMDD mutants were observed in six of the nine individuals in the VBT group and none were observed in the non-VBT group (67% vs 0%, *P* < 0.01). All patients had HBV genotype C. The frequency of precore and core promotor variants did not differ significantly between VBT and non-VBT groups.

Patient 6 in the VBT group had a precore mutation in the 1896 position and was HBeAg-positive (Table 3). Patients 3 and 8 also had smaller A peaks than G peaks at the 1896 position, but were classified as wild-type (Table 3).

VBT relative to baseline HBV DNA levels

When all factors, including gender, duration of infection, baseline HBV DNA, HBV DNA at the 6th month of treatment, and baseline ALT in HBeAg-positive CHB patients were analyzed by logistic regression, the most important factor associated with VBT was only baseline HBV DNA level (*r*² = 0.12, *P* < 0.05).

When HBeAg-positive CHB patients were divided into two groups by the point of 6.6 log HBV DNA level: one group > 6.6 log HBV DNA; and other group \leq 6.6 log HBV DNA, there was a significant difference between two groups in terms of VBT rate (*P* < 0.05). We also compared the cumulative VBT rate between these two groups according to the treatment duration with Kaplan-Meier analysis. The VBT percentage in HBeAg-positive hepatitis patients with HBV DNA levels \leq 6.6 log₁₀ (*n* = 15) was 6.7%, 18%, and 39% at 1, 2, and 3 years, respectively, while that in patients with HBV DNA levels > 6.6 log₁₀ (*n* = 17) was 19%, 45%, and 88% at 1, 2, and 3 years, respectively (*P* = 0.061) (Figure 1).

The next important factor associated with VBT was HBV DNA at the 6th month of treatment, but it did not show a significant correlation with VBT. In HBeAg-negative patients, no relationship between the VBT rate and HBV DNA level was observed.

DISCUSSION

Our study demonstrated that patients with lower HBV DNA levels had less VBT with LAM therapy. Other predictors of LAM-resistant mutations include male gender, Asian ethnicity, higher baseline HBV DNA, longer duration of lamivudine treatment, and higher BMI^[2]. Our study clearly showed that the baseline HBV DNA level is the most significant predictor. However, it is true that the number of study subjects was relatively small (*n* = 79) and the median duration of follow-up was about 40 mo.

But, the findings of our study are contradictory to the observation of the Taiwanese study^[8]. They reported that HBeAg status, HBV DNA, ALT levels and treatment duration were the major determinants for the YMDD mutation during lamivudine therapy. The most important difference between two studies is what they observed. We observed VBT, but they observed the genotypic resistance. Besides, there were several differences between two studies, such as study designs and the test for HBV DNA.

Table 2 Response to lamivudine before breakthrough (mean \pm SD)

	HBeAg-positive hepatitis (n = 34)			HBeAg-negative hepatitis (n = 45)		
	All patients (n = 34)	BT (n = 14)	No BT (n = 20)	All patients (n = 45)	BT (n = 10)	No BT (n = 35)
Max suppression (log ₁₀ copies/mL)	4.7 \pm 1.4	4.8 \pm 1.7	4.6 \pm 1.2	4.3 \pm 1.6	4.3 \pm 1.7	4.3 \pm 1.6
Initial suppression (log ₁₀ copies/mL)	4.2 \pm 1.7	4.2 \pm 2.0	4.3 \pm 1.5	3.3 \pm 2.1	2.8 \pm 2.5	3.4 \pm 1.9
Patients with IVR (%)	80	75	83	81	67	85
Patients at 6 mo biochemical response (%)	90	82	94	84	86	83
Patients at 12 mo biochemical response (%)	96	100	93	82	80	82
Time to first detection of BT	-	26 \pm 10	-	-	32 \pm 12	-

BT: Breakthrough; Max suppression: Maximal virologic suppression; Initial suppression: Initial virologic suppression; IVR: Initial virologic response at 6 mo; Biochemical response: ALT normalization.

Table 3 HBV genotypes, variants, and lamivudine-resistant mutants

Patients with VBT				During BT							
Patient	Sex	BT	Genotype	Month after LAM	ALT	HBeAg	HBV DNA log ₁₀ copies/mL	PC	CP A1763T G1765A	L180M	M204I/V
1	F	+	C	19	90	-	5.4	Wild	TA	M	I/V
2	M	+	C	18	29	-	3	Stop	TA	L	I/V
3	F	+	C	12	12	+	6.8	Wild	AG	L	M
4	M	+	C	18	44	+	8.3	Wild	TA	M	I/V
5	M	+	C	36	NA	+	5.2	Wild	TA	M	I/V
6	F	+	C	21	42	+	5.7	Wild	AG	L	M
7	M	+	C	27	24	+	6.6	Stop	TA	M	I/V
8	F	+	C	21	NA	+	6.7	Wild	AG	L	M
9	F	+	C	30	108	+	6.7	Wild	TA	M	I/V
Patients without VBT				End of follow-up							
Patient	Sex	BT	Genotype	Month after LAM	ALT	HBeAg	HBV DNA log ₁₀ copies/mL	PC	CP A1763T G1765A	L180M	M204I/V
1	M	-	C	9	28	+	4.6	Wild	TA	L	M
2	M	-	C	30	NA	+	5.4	Wild	TA	L	M
3	M	-	C	32	12	-	1	UD	UD	L	M
4	M	-	C	27	NA	+	1	Wild	TA	L	M
5	M	-	C	36	42	-	1	UD	UD	L	M
6	M	-	C	24	24	+	1	Wild	TA	UD	UD

PC: Precore mutants; CP: Core promotor mutants; SR: Suboptimal response; NA: Not available; UD: Undetectable; LAM: Lamivudine; BT: Breakthrough; VBT: Virologic breakthrough.

When the study criteria were set up, AGA (American Gastroenterology Association) expert panel recommendations were followed; HBeAg-positive patients were individuals with HBV DNA > 5 log₁₀ copies/mL, while HBeAg-negative patients were individuals with HBV DNA > 4 log₁₀ copies/mL. In this study, a lower HBV DNA cutoff, above 3 log, was used for starting antiviral treatment. Although we did not know the recent Taiwanese data when we started to treat our patients, but it shows that higher baseline serum DNA levels are associated with increased risk of HCC and liver cirrhosis independent of serum ALT level^[9]. The AGA panel specifies that ALT levels should be abnormal, but this is not always helpful in determining who should be treated^[10]. ALT levels do not serve as a good indicator of liver cirrhosis. In addition, revision of the normal limits for ALT levels was recommended in patients with chronic HCV infection or nonalcoholic fatty liver disease (NAFLD) since current standards for normal were defined using the populations that included individuals with subclinical disease^[11,12]. Some patients experienced disease progression during follow-up because they had normal ALT levels and were not treated

earlier. Thus, early antiviral treatment may be beneficial to reduce HBV DNA levels, HCC risk, and the frequency of liver transplantations^[13]. LAM can reduce serum HBV DNA levels and normalize ALT level in cirrhotic patients^[14,15].

As shown in Table 1, we noted that HBeAg-negative CHB have lesser LAM resistance than HBeAg-positive CHB, which is in agreement with previous studies^[16,17].

We conducted genotypic resistance analysis on 15 patients who had serum samples harvested upon viral breakthrough or at the end of follow-up. Genotypic resistance occurred only in the VBT group and was not observed in the non-VBT group. However, three patients in the VBT group, and five in the non-VBT group had the wild-type. The most plausible explanation for the disparity between phenotypic and genotypic resistance is medication non-compliance. Possible solutions for this problem, like pill counts and nurse monitoring, were beyond the scope of our study. A blip in viral numbers is another potential explanation for the presence of wild-type virus in the VBT patients. The InnoLiPa assay could be used to define the mixed strains in six patients from the VBT group. When

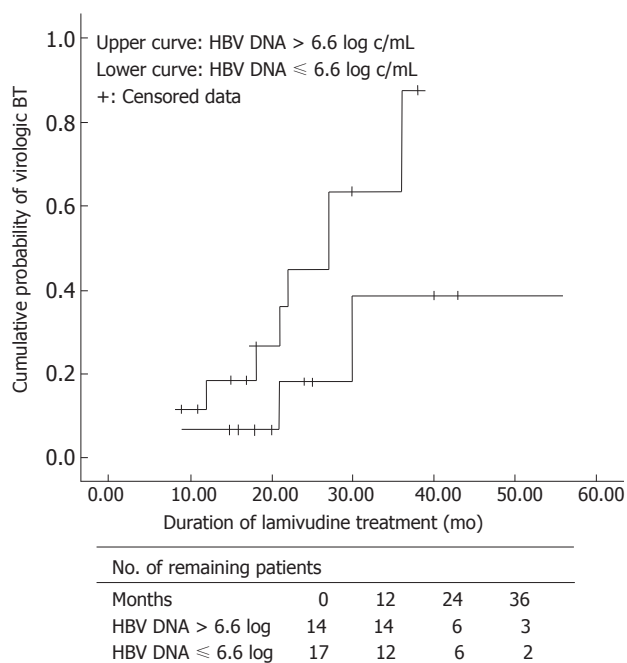


Figure 1 Cumulative probability of breakthrough. The cumulative rate of breakthrough tended to differ between HBV DNA > 6.6 log and HBV DNA ≤ 6.6 log group ($P = 0.06$). The cumulative rate was 88% and 39% after 3 years of lamivudine treatment in HBV DNA > 6.6 log and HBV DNA ≤ 6.6 log group, respectively.

we found the viral breakthrough in 25 patients, we recommended several options such as adefovir monotherapy, adefovir overlapped with lamivudine, and tenofovir monotherapy to our patients.

Two patients of suboptimal responders had YMDD mutants. They did not show VBT during treatment period. But we might have missed VBT in these patients because the test for HBV DNA had not been tested at the right time, or YMDD mutants may have existed even before LAM monotherapy^[18].

The experimental results from both direct sequencing and InnoLipa DR2 line probe assay were consistent in all patients.

The monthly U.S. wholesale prices for hepatitis B treatment are \$158 for lamivudine (100 mg/d), \$528 for adefovir (10 mg/d), \$715 for entecavir (0.5 mg/d), \$1429 for entecavir salvage therapy (1 mg/d), and \$1540 for peginterferon alpha-2a (180 µg/wk). Thus, the most cost-effective regimen across most healthcare settings, independent of HBeAg status, is LAM monotherapy, and adefovir salvage therapy for individuals with LAM resistance^[4].

Our study was limited by the small patient sample size as well as potential referral bias. We were unable to conduct genotypic resistance analysis for all subjects since serum samples were not always available.

We observed the correlation between the occurrence of VBT and initial HBV DNA level. We suggest that 6.6 log₁₀ in HBeAg-positive CHB patients can be a criterion for long-term LAM monotherapy and the patients with baseline HBV DNA level ≤ 6.6 log may derive the most benefit from LAM monotherapy because of low incidence of VBT. For individuals with CHB, who have a baseline

HBV DNA ≤ 6.6 log and limited financial means, we conclude that LAM remains an affordable and effective therapy.

ACKNOWLEDGMENTS

Dr. Hann is on the speaker's bureau for Gilead, Bristol-Myers Squibb and GlaxoSmithKline, and has received research support from all three companies. The authors are grateful to Dr. Scott Fung at the University of Toronto for his valuable advice in preparing the manuscript, Ms. Munira Hussain at the University of Michigan for providing invaluable information about primers and the PCR protocol, and Dr. Mark Feitelson for providing the laboratory facility to conduct the molecular biology.

COMMENTS

Background

Lamivudine is the first FDA-approved oral antiviral agents for chronic hepatitis B (CHB) with 10 years experience of safety and efficacy. It was recently excluded from the 1st line drugs in 2007 AASLD practice guideline because of its high rate of drug resistance. We investigated the factors associated with virologic breakthrough in patients on lamivudine therapy and identified a subgroup of patients who responded well to lamivudine without developing virologic breakthrough. Therefore, we believe that for this subgroup of CHB patients, lamivudine may still remain the most cost-effective drug among 4 currently available oral anti-HBV drugs.

Research frontiers

Other predictors of lamivudine resistance include male gender, Asian ethnicity, higher baseline HBV DNA, long duration of lamivudine treatment, and higher BMI.

Innovations and breakthroughs

Our study clearly showed that the baseline HBV DNA level is the most significant predictor for virologic breakthrough and identified the discriminating number as 6.6 log₁₀ HBV DNA.

Applications

Physicians can apply this knowledge to predict lamivudine resistance and may start lamivudine for this group of patients who present with baseline HBV DNA level lower than 6.6 log.

Terminology

Virologic breakthrough: > 1 log₁₀ copies increase in HBV DNA from nadir on two consecutive occasions after an initial virologic response.

Peer review

The authors investigated several clinical, biochemical and virologic factors, including gender, route of infection, duration of infection, baseline HBV DNA level, baseline ALT level, and other factors in HBeAg-positive chronic hepatitis B patients. The study is well conducted and the results indicate that the most important predictor of virologic breakthrough is the baseline HBV DNA.

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S- Editor Zhu LH L- Editor Kumar M E- Editor Wang HF

Bravo (wireless) ambulatory esophageal pH monitoring: How do day 1 and day 2 results compare?

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Received: 2007-03-08 Accepted: 2007-04-26

2007; 13(30): 4091-4095

<http://www.wjgnet.com/1007-9327/13/4091.asp>

Abstract

AIM: To investigate if differences exist for patients' gastroesophageal reflux as measured by the Bravo ambulatory esophageal pH system between d 1 and d 2.

METHODS: A retrospective study of 27 consecutive adult patients who underwent Bravo esophageal pH monitoring was performed. Patients underwent EGD under IV conscious sedation prior to Bravo placement. Acid reflux variables and symptom scores for d 1 were compared to d 2.

RESULTS: The mean doses of fentanyl and midazolam were 90.4 µg and 7.2 mg, respectively. D 1 results were significantly more elevated than d 2 with respect to total time pH < 4, upright position reflux, and mean number of long refluxes. No statistical difference was noted between the two days for supine position reflux, number of refluxes, duration of longest reflux, episodes of heartburn, and symptom score.

CONCLUSION: Patients undergoing Bravo esophageal pH monitoring in association with EGD and moderate conscious sedation experience significantly more acid reflux on d 1 compared to d 2. The IV sedation may be responsible for the increased reflux on d 1. Performed this way, 48-h Bravo results may not be entirely representative of the patients' true GE reflux profile.

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Key words: Esophageal pH monitoring; Bravo; Gastroesophageal reflux disease; Reflux; pH testing

Bechtold ML, Holly JSL, Thaler K, Marshall JB. Bravo (wireless) ambulatory esophageal pH monitoring: How do day 1 and day 2 results compare? *World J Gastroenterol*

INTRODUCTION

Ambulatory esophageal pH monitoring has for many years been a widely available method for quantifying esophageal acid exposure in patients with suspected gastroesophageal reflux disease (GERD)^[1-3]. Until recently, the only way to perform ambulatory esophageal pH monitoring has been by the transnasal placement of a pH catheter probe, left in place for 24 h. Indications have included evaluation prior to anti-reflux surgery, refractory symptoms despite proton pump inhibitor (PPI) therapy, recurrent GERD symptoms following anti-reflux surgery, and continuation of atypical or extra-esophageal symptoms of GERD despite empirical therapy with a PPI.

At best, ambulatory pH monitoring with a catheter pH probe is moderately uncomfortable to patients, and there are some patients who are unable to tolerate the catheter altogether. Performance in adults has also traditionally required that esophageal manometry first be performed to localize the position of the lower esophageal sphincter relative to the nares. In addition, patients with nasally passed pH catheters often restrict their activities and diet, which has the potential to underestimate the amount of reflux they might have under more normal circumstances^[2,4]. These various limitations of catheter-based ambulatory pH monitoring were major factors that led to the development of the Bravo pH monitoring system (Medtronic, Shoreview, MN), a catheter-less system that employs a radiotelemetry pH-sensing capsule clipped to the esophageal wall, which transmits pH data to a recorder worn by the patient. The Bravo delivery system was designed for either oral or nasal passage^[5], though the capsule size may make transnasal passage difficult for some patients^[6]. Compared to catheter-based pH monitoring, Bravo esophageal pH monitoring is better tolerated by patients and permits increased duration of pH recording^[6-9].

Some of the technical details of Bravo pH capsule placement have not been standardized. For instance, the Bravo capsule may be clipped to the esophagus at the time that a patient undergoes diagnostic EGD utilizing conscious sedation. Alternatively, placement may be done on a different day, potentially without sedation, provided that the distance from the incisors (or the nares) to the GE

junction is known based on measurements from previous esophageal manometry or endoscopy.

Most studies have examined Bravo esophageal pH results over an entire 48-h period without mentioning or giving specific d 1 and d 2 results. The few studies examining differences in d 1 and d 2 have varied results. Since patients in our practice undergo concomitant EGD under moderate sedation, we wondered if this sedation could potentially affect the d 1 results, possibly increasing gastroesophageal reflux on d 1. Our study compared d 1 with d 2 Bravo esophageal pH results.

MATERIALS AND METHODS

We conducted a retrospective study of 27 consecutive patients age 15 years and older at the University of Missouri Hospital and Clinics who underwent 48-h Bravo ambulatory esophageal pH monitoring for suspected GERD off of any anti-reflux medications. The study was approved by the Institutional Review Board of our institution. All patients had stopped any proton pump inhibitors and H₂-receptor antagonists seven days before placement of their Bravo pH capsules.

All patients in our study underwent EGD under IV conscious sedation with fentanyl and midazolam on the same day just prior to Bravo pH capsule placement. Four different endoscopists did the EGDs and Bravo placements. The amount of sedation given patients was at the discretion of the endoscopist. The Bravo capsule was placed 6 cm above the gastroesophageal junction in standard fashion^[5,10]. We utilized vacuum suction applied at > 510 mmHg for 30 s prior to clipping the Bravo capsule. Immediately after deployment, direct visualization of the attached capsule was performed with the endoscope to confirm proper placement. Patients were instructed to wear the Bravo data recorder around their waist or to be within five feet of it at all times during the 48-h recording period. After recovery from their conscious sedation and discharge from our GI laboratory, patients were encouraged to resume their normal daily activities and usual diets. Patients were instructed to keep a diary to document when they ate, periods of sleep, and the occurrence of symptoms. They were also told to press a button on the data recorder when they perceived GERD-related symptoms.

After 48 h, patients returned to the GI laboratory, where they turned in their receivers and diaries. The data contained in the receiver was then downloaded to a computer, analyzed by Medtronic software, which then generated a summary report, which was reviewed and interpreted by one gastroenterologist (JBM). The computer analysis gave the percent of total time pH < 4, percent of upright time pH < 4, percent of supine time pH < 4, total number of reflux episodes, number of reflux episodes 5 min or longer, and the duration of the longest reflux episode for the entire 48-h recording period for d 1 and d 2 separately. A symptom score was calculated by dividing episodes of heartburn (or other reflux symptoms) which correlated with pH < 4 by the total number of heartburn episodes (or other reflux symptoms), and compared between d 1 and d 2.

Statistical analysis

Age of patients and doses of sedative medications were reported as mean \pm SD. The reflux data collected from d 1 were statistically compared to d 2 to examine for significant differences. Differences were calculated for each variable comparing d 1 minus d 2. By this method, a positive value for the difference indicated that the value for d 1 was higher than the value for d 2. Since some of the measured variables were not normally distributed, the nonparametric Wilcoxon Signed Rank test was used to test the null hypothesis of no difference in the responses for the two days. In view of the large number of tests conducted, results were considered significant only for *P* values < 0.01. Ninety-five percent confidence interval estimates of the median difference between days were calculated. Analyses were done using the statistical software SAS (SAS Institute Inc., Cary, NC, USA).

RESULTS

Twenty-seven consecutive patients met entry criteria into the study (i.e. Bravo pH study done for suspected GERD with the study done off of anti-reflux medications). One patient was excluded after data analysis showed that their Bravo capsule had dislodged from the esophagus right after it had been placed. That left 26 patients for analysis as part of the study. This included six males (23%) and twenty females (77%), with a mean age of 47.6 ± 12.2 (range 15 to 67) years. Only three of the patients were less than 35 years of age (ages 15, 24 and 29 years). Five of the patients had a prior Nissen fundoplication, and one had a prior gastropasty. The mean doses of sedative medications used in the patients were 90.4 ± 24.6 μ g of fentanyl and 7.2 ± 2.8 mg of midazolam.

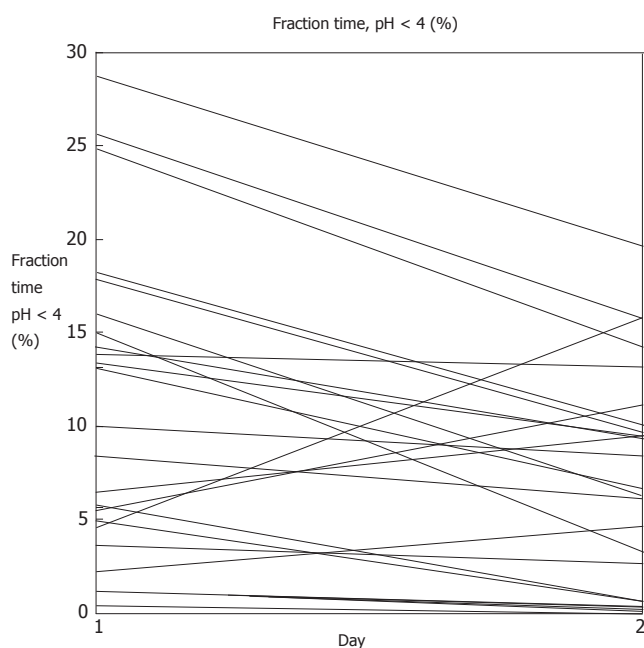
Table 1 shows the d 1 and d 2 results. Of the ten variables examined, significant differences (*P* < 0.01) were seen in three. More reflux was seen on d 1 as compared to d 2 as regards percent of total time pH < 4 (*P* = 0.0049), percent of upright time pH < 4 (*P* = 0.0051), and the number of long reflux episodes (*P* = 0.0077). There were trends to more reflux on d 1 compared to d 2 in terms of the mean number of reflux episodes per day and the mean duration of long reflux episodes, but these differences were not statistically significant. There were no differences between d 1 and d 2 in terms of percent of supine time pH < 4, number of heartburn episodes, and symptom scores. Figure 1, Figure 2, Figure 3 plot the d 1 and d 2 values for the three variables that showed statistical differences.

DISCUSSION

Since its introduction in 1974, ambulatory esophageal pH monitoring has helped to advance our knowledge regarding GERD, and has become an important tool in its diagnosis and management^[1,11]. Until recently, the performance of the test has required the transnasal placement of a pH catheter probe, left in place for 24 h. In addition to being an uncomfortable test, patients commonly restrict their activities and diet, which has the potential to underestimate the amount of reflux that might occur under more typical

Table 1 Bravo data analysis using mean differences for d 1-d 2 and medians with 95% confidence intervals

Variable	d 1 (mean)	d 2 (mean)	P-Value	95% CI
Total time pH < 4 (%)	10.4	7.1	0.0049	0.8-8.0
Total time pH < 4-Upright (%)	11.7	6.3	0.0051	1.6-7.9
Total time pH < 4-Supine (%)	9.4	10.0	0.8596	
Mean number of reflux episodes (<i>n</i>)	65.3	55.6	0.0257	
Mean number of long refluxes (<i>n</i>)	6.8	3.8	0.0077	0.0-6.0
Mean duration of long refluxes (min)	26.5	19.7	0.0617	
Total heartburn episodes (<i>n</i>)	6.2	8.0	0.2752	
Heartburn episodes with pH < 4 (<i>n</i>)	2.5	2.9	0.686	
Symptom score	0.323	0.254	0.1353	

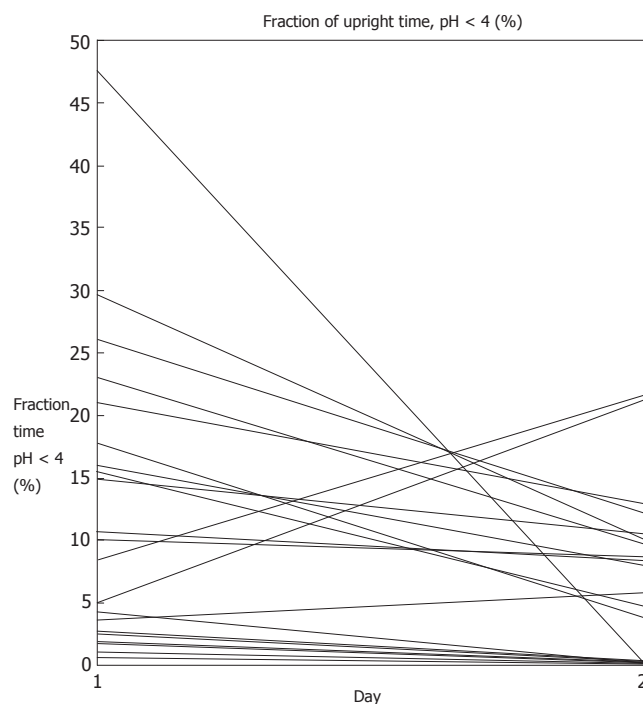
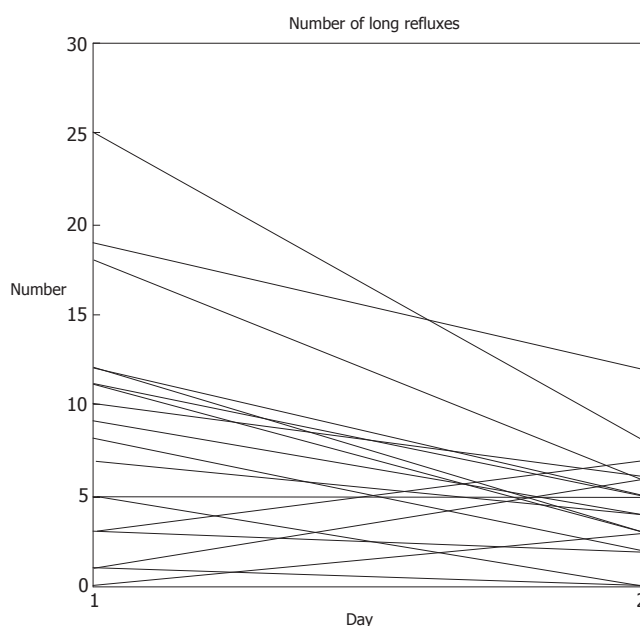
**Figure 1** Individual comparison of the fraction of total time of pH < 4 between d 1 and d 2.

lifestyle circumstances^[2,4].

The recently introduced Bravo pH system, which uses a radiotelemetric capsule that is clipped to the esophageal wall, has been shown to be better tolerated by patients and to permit a more normal lifestyle during the study period^[6,8,9,11]. Acid reflux monitoring results with Bravo should better reflect the real pattern of reflux in the day-to-day life of patients. The other potential major advantage of Bravo technology is that it permits longer periods of monitoring. While most Bravo studies currently are 48 h in duration, some have reported monitoring periods for as long as four days^[12].

One of the most pressing issues relating to Bravo esophageal pH testing is that normal values are much less well established for this new technology as compared to conventional catheter-based pH monitoring^[11]. Another relates to the lack of standardization as to whether the tests are performed without sedation on a day separate from endoscopic exams versus placing the pH capsule in association with EGD.

In our own practice, we have placed the Bravo capsule in association with the patient undergoing an EGD under

**Figure 2** Individual comparison of the fraction of total time of pH < 4 in upright position between d 1 and d 2.**Figure 3** Number of long refluxes comparison between d 1 and d 2.

moderate conscious sedation on the same day. Because of concern that d 1 was therefore not typical of our patients' usual lifestyle, we decided to examine the amount of gastroesophageal reflux that our patients experienced on d 1 *versus* d 2. We hypothesized that patients might have more reflux on d 1 because of the effects of the endoscopy and sedation. Our findings confirmed this, showing significantly more reflux on d 1 than d 2 in terms of percent of total time pH < 4, percent of upright time pH < 4, and the number of long reflux episodes. There was no statistical difference in the supine time pH < 4,

absolute number of reflux episodes per day, mean duration of long reflux episodes, number of heartburn episodes, and symptom scores. While we believe that the moderate intravenous sedation we gave to our patients to be responsible for the greater reflux on d 1, our study was not designed to clarify the specific mechanism. Our patients received a mean dose of 90 µg of fentanyl and 7 mg of midazolam. While we did see more upright reflux on d 1 compared to d 2, there was no difference in supine reflux between the two days. A potential explanation for this observation is that the effects of their sedation may just be limited to the first 8 or 12 h after the endoscopy and Bravo capsule placement. Consequently, the medication effects may have worn completely off by the time the patient goes to bed that first night.

While most studies employing Bravo technology have reported overall 48-h pH results, some have compared d 1 with d 2. These latter studies have shown greatly varying findings. Similar to our study, Bhat *et al*^[13] found more reflux on d 1 than d 2. Increased esophageal acid exposure during first six hours after capsule attachment was believed to have accounted for this finding. As in our series, patients in their study underwent EGD under conscious sedation just preceding Bravo placement, though they did not report specific medication dosages.

Most of the studies which have commented on d 1 *versus* d 2 reflux have reported no consistent differences. For instance, Prakash *et al*^[7] said there were no differences in reflux variables between d 1 and d 2, though they did not report specific values. Patients in their study underwent EGD under conscious sedation, employing a variety of different medications, at the time of Bravo placement, though specific medication doses were not reported. Pandolfino *et al*^[2] found no differences between d 1 and d 2 in their patients who also underwent same-day EGD with 50 to 75 mg of meperidine and 1 to 5 mg of midazolam. Tseng *et al*^[14] found no consistent differences in their patient group who underwent same-day EGD with fentanyl and midazolam, though sedation doses were not given. Ahlawat *et al*^[15] found no consistent differences in their group of patients who underwent same-day EGD under propofol.

Interestingly, one study found more reflux on d 2 than d 1^[16]. Patients in this study did not undergo EGD or receive sedation on the day of their Bravo placements. Another study reported more reflux episodes on d 2 than d 1, but no consistent differences in other reflux variables^[8]. Patients in this latter study underwent EGD on the day of Bravo placement, but no mention was given as to whether patients received sedation or the dose of sedative medications.

What should we make of the great variability that different studies have found between d 1 reflux *versus* d 2 reflux? For one thing, it points out that we need more study of the effects of the various protocols used to place Bravo pH capsules, and how they impact the d 1, d 2 and overall reflux results. It is entirely possible that there will be different normal ranges for reflux depending on whether the Bravo capsule is placed at the time of EGD with sedation versus it being placed without same-day endoscopy and sedation. Perhaps more emphasis

should be given to placing the Bravo without endoscopy and sedation altogether. Alternatively, if endoscopy and sedation are to be used, we could look into doing longer studies, say 72 or 96-h studies, and then eliminating the d 1 data.

In conclusion, patients undergoing Bravo (wireless) ambulatory esophageal pH monitoring in our study showed increased acid reflux in the distal esophagus on d 1 as compared to d 2. We believe that this was the result of patients having undergone EGD with moderate sedation just prior to Bravo placement. However, given the variability as to what the literature reports in regards to d 1 *versus* d 2 reflux results, we believe that much more study of this phenomenon is needed as we apply the test clinically. We also believe that better establishment of normal results for Bravo pH monitoring is needed and will need to reflect the placement protocol used.

ACKNOWLEDGMENTS

The authors thank Richard W Madsen, PhD for statistical support.

COMMENTS

Background

The recently introduced Bravo ambulatory esophageal pH system offers a catheter-less method of monitoring gastroesophageal (GE) reflux over an extended period and of correlating symptoms with acid reflux episodes. Limited data is available comparing d 1 with d 2 results. However, since patients may be sedated to clip the pH electrode to the esophagus, this sedation may potentially affect the d 1 results, possibly increasing GE reflux on d 1. This study compared d 1 with d 2 results.

Research frontiers

The Bravo ambulatory esophageal pH system is a relatively new technology used to analyze GERD. This new system allows for 48 h of monitoring and is more comfortable to patients as compared to the 24 h transnasal pH catheter probe. However, limited data has suggested the results of the Bravo system vary over the two-day span. This study examines if a difference does exist in the patients' reflux profile from d 1 to d 2 and postulates potential contributing factors.

Innovations & breakthroughs

The Bravo ambulatory esophageal pH system is a catheter-less system which is becoming more popular for the analysis of GERD.

Applications

This article demonstrates a significant difference in total time pH < 4, upright position reflux, and mean number of long refluxes between d 1 and d 2. The most likely contributing factor was the use of IV sedation prior to the attachment of the pH probe. Future studies may be performed to compare the Bravo capsule placement with and without prior IV sedation.

Peer review

This retrospective study explores the effect of conscious sedation, performed before endoscopy and capsule placement, on 48-h Bravo pH findings. The Authors observed higher values of total acid exposure, upright reflux and number of long refluxes on d 1 vs d 2. Based on these retrospective data and on literature data, these differences may result from conscious sedation. Rationale and objectives of this study are very interesting, methods and presentation of data are reliable and allow relevant scientific conclusions.

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S- Editor Zhu LH L- Editor Negro F E- Editor Wang HF

RAPID COMMUNICATION

Low-dose tenofovir is more potent than adefovir and is effective in controlling HBV viremia in chronic HBeAg-negative hepatitis B

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Received: 2007-04-23 Accepted: 2007-05-28

chronic HBeAg-negative hepatitis B. *World J Gastroenterol* 2007; 13(30): 4096-4099

<http://www.wjgnet.com/1007-9327/13/4096.asp>

Abstract

AIM: To study the efficacy of tenofovir disoproxil fumarate (TDF) at low dose in a small open trial of chronic hepatitis B patients with advanced stage disease.

METHODS: Eleven patients were treated with TDF 75 mg for a median period of 80 (range, 24-576) wk and then 7 cases were shifted to an adefovir 10 mg treatment group. All patients had been pre-treated with lamivudine: 5 had YMDD resistant mutants and 6 wild-type virus. When TDF was started, 4 patients had low-level viremia and 6 were PCR-negative.

RESULTS: During TDF treatment, PCR remained negative in 10 patients, transaminase levels were normal and no significant viral breakthrough was observed. The drug was well tolerated in all cases. When TDF 75 mg was substituted with adefovir 10 mg, 3 out of 7 patients had a persistent viral rebound (2700-130 000 copies/mL), in whom lamivudine had to be reintroduced.

CONCLUSION: Low-dose TDF monotherapy can control HBV viremia for an extended period of time without the emergence of resistance and is more potent than adefovir at the standard dosage. The use of a reduced dose of TDF could diminish the cost of therapy in low-income countries, but further studies in a larger population and in HBeAg-positive subjects are needed.

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Key words: Tenofovir; Chronic Hepatitis B; Adefovir; Nucleotide analogues; Low-income countries

Del Poggio P, Zaccanelli M, Oggionni M, Colombo S, Jamoletti C, Puhalo V. Low-dose tenofovir is more potent than adefovir and is effective in controlling HBV viremia in

INTRODUCTION

Tenofovir disoproxil fumarate (TDF) is a nucleotide analogue approved for the treatment of HIV infection and is structurally related to adefovir. TDF is also active against hepatitis B virus (HBV) and is equipotent to adefovir *in vitro*^[1,2], but because of its lower nephrotoxicity, it can be used at higher dosage (e.g. 300 mg/d) and is more active than its parent compound against HBV *in vivo*^[3-5]. TDF is equally effective against wild-type and lamivudine-resistant HBV^[6] in HIV-coinfected patients^[7], and may in the future replace adefovir in the therapy of hepatitis B. The main concern with TDF is the lack of safety data on the absence of nephrotoxicity of the 300 mg dose in long-term treatment. Although registration and large retrospective studies in HIV infection^[8-10] have shown that the risk of deterioration of renal function is less than 3%, this problem could limit the use of TDF in decompensated cirrhotics with borderline renal function. The dose of 300 mg was chosen because these studies were conducted in coinfected patients with a dosage active against HIV, but there are no data on the susceptibility of HBV to lower TDF doses. Adefovir *per se* was initially used at 60-120 mg/d in order to achieve a significant HIV inhibition^[11], a dosage well above the 10 mg dose approved in the treatment of hepatitis B. It is therefore feasible that TDF, like its parent drug adefovir, could be used at lower dosage. Another potential advantage of low-dose TDF would be the reduction of the cost of therapy in low-income countries, where the death toll of hepatitis B is worrisome. In consideration of this, we decided to test the efficacy of low-dose TDF in a small open trial of chronic HBeAg-negative hepatitis B patients with advanced stage disease.

MATERIALS AND METHODS

Eleven patients with chronic HBeAg-negative hepatitis B (7 males and 4 females; median age 63 years, range 40-77 years) were included in this study. The characteristics of the patients are described in Table 1. Nine had biopsy-

Table 1 Baseline characteristics of the patients

Patient	Sex	Age (yr)	Liver biopsy	Complications	HBV DNA (pg/mL) ¹	LAM ³ (wk)	YMDD
1	F	77	Cirrhosis	HCC	8	96	M204I
2	M	40	CAH Ishak 9;2		2	156	M204V
3	M	63	Cirrhosis		Undetectable ²	188	M204V
4	F	68	Cirrhosis	F2 esoph varices	1600	44	M204I
5	M	55	Cirrhosis		Undetectable ²	104	M204V, L180M
6	F	58	Cirrhosis		450	88	WT
7	M	68	Cirrhosis	F2 esoph varices	64	76	WT
8	M	57	CAH Ishak 7; 4		162	116	WT
9	M	65	Cirrhosis		78	88	WT
10	M	58	Cirrhosis		0.2	52	WT
11	F	75	Cirrhosis		3,100	116	WT

¹Basal HBV DNA (Digene Capture 2 Assay, sensitivity 0.2 pg/mL, equal to 105 copies/mL) before starting lamivudine; ²Sample taken after ALT flare; ³Duration of lamivudine treatment before starting tenofovir; esoph: esophageal.

proven well compensated cirrhosis with preserved hepatic function (Child-Pugh A) and two chronic active hepatitis with mild-moderate fibrosis, but with frequent ALT flares to more than 10 times normal value. Two patients had F2 oesophageal varices without previous episodes of bleeding and were on primary prophylaxis with Nadolol. One patient had a unifocal small hepatocellular carcinoma that was treated with percutaneous alcohol injection, resulting in a complete necrosis of the nodule. None of them had ascites, although one was on diuretic treatment with Canrenoate 100 mg daily orally. There were no comorbidities and the body mass index was under 30% in all cases. All 11 patients had been pre-treated with lamivudine 100 mg daily for a median duration of 96 (range 44-188) wk and 5 had developed mutations in the YMDD motif. Four had a single mutation (M204V in 2 cases and M204I in the other 2), one patient had a double mutation (M204V and L180M). The presence of YMDD resistance mutants was detected with the Innolipa assay (Innolipa Line Probe Assay, Innogenetics). At the time of emergence of resistance, 4 patients had normal levels of alanine aminotransferase (ALT) and negative serum branched HBV DNA (Versant HBV DNA 3.0 Bayer, sensitivity < 2000 copies/mL), although viral DNA could be detected by polymerase chain reaction (Innolipa Assay, Innogenetics with a sensitivity lower than 1000 copies/mL). One patient with a YMDD mutant had elevated ALT and serum HBV DNA of 4.8×10^6 copies/mL. The remaining 6 patients did not develop YMDD mutants and were PCR-negative with normal ALT during the entire treatment with lamivudine. They were shifted to TDF to prevent the emergence of YMDD resistant mutants, because at the time adefovir was not yet available to rescue the patients in case of development of lamivudine resistance. In all patients, lamivudine was withdrawn abruptly and substituted with TDF 75 mg daily orally, with no washout period. Tenofovir was given off label, with the consent of the patients, and was continued for a period of 80 (range 24-144) wk.

Table 2 Results of treatment with tenofovir disoproxil fumarate (TDF) in the individual patients

Patient (YMDD/WT)	Basal HBV DNA ¹ (cp/mL)	Duration of treatment with TDF (wk)	HBV DNA (end of TDF) (cp/mL)	Drug substituted for TDF	HBV DNA after substitution of TDF
1 (YMDD)	1000-2000	96	< 1000	ADV	130,000 cp/mL
2 (YMDD)	4.8×10^6	144	< 1000	ADV	No viral rebound
3 (YMDD)	1000-2000	56	< 1000	LAM	No viral rebound
4 (YMDD)	1000-2000	80	< 1000 ²	ADV + LAM	No viral rebound
5 (YMDD)	1000-2000	60	< 1000	ADV	No viral rebound
6 WT	< 1000	108	< 1000	ADV	No viral rebound
7 WT	< 1000	76	< 1000	ADV	21 000 cp/mL
8 WT	< 1000	88	< 1000	ADV	2700 cp/mL
9 WT	< 1000	116	< 1000	ADV	No viral rebound
10 WT	< 1000	52	< 1000	LAM added to TDF	No viral rebound
11 WT	< 1000	24	< 1000	LAM	No viral rebound

¹At the end of lamivudine treatment and before starting TDF; ²After 8 mo of TDF, HBVDNA was unchanged but YMDD disappeared. Lamivudine was then added to TDF with prompt decrease of DNA below 1000 cp/mL.

When adefovir became available in the country, TDF was substituted with the former in 7 cases, with lamivudine alone or in combination with a nucleotide in the other 4 cases.

RESULTS

The results are shown in Table 2. Four of the 5 patients with YMDD mutants, including the patient with high viremia, became HBV DNA-negative by PCR assay after 8-24 wk of tenofovir treatment and remained negative throughout the entire period of treatment. One patient, who was HBV DNA-negative with the bDNA assay and PCR-positive at baseline, did not become PCR-negative during tenofovir treatment. After 32 wk of tenofovir monotherapy, she cleared the YMDD mutant and the wild-type virus reappeared, lamivudine was then reinstituted in addition to tenofovir, and subsequently she achieved PCR negativity. The six patients without lamivudine resistance were shifted to the tenofovir treatment group when their serum HBV DNA was undetectable by PCR and remained negative throughout the whole period of tenofovir treatment, with the exception of a transient viral blip in two cases at week 88 and 96 (5500 and 7500 copies/mL, respectively). Transaminase levels remained normal in all patients. The drug was well tolerated and no side effects were reported, in particular serum creatinine remained within normal limits in all cases. In 7 patients, tenofovir was substituted with adefovir 10 mg/d and a viral rebound was observed in 3 cases, one with previous lamivudine resistance and 2 with wild-type virus. The viral rebound was greater than 3 log in all the 3 cases and was controlled only after the addition of lamivudine to adefovir with

HBV DNA again undetectable by PCR. In an other 2 cases, lamivudine monotherapy was reinstituted after stopping tenofovir and no viral rebound was observed, but one patient died of hepatocellular carcinoma 96 wk after the reinstitution of lamivudine. One patient is still on tenofovir, but lamivudine was added after 52 wk to prevent the emergence of resistance to tenofovir and the patient is still PCR-negative at a follow-up of 100 wk.

DISCUSSION

In our small series of patients, a dose of TDF as low as 75 mg daily was able to suppress HBV viremia of both wild-type and lamivudine-resistant virus for a median period of 80 wk. All the patients were cirrhotic and/or with severe hepatitis and all achieved a good control of viremia, becoming PCR-negative with the Innolipa test, which has a sensitivity lower than 1000 copies/mL and in the range of 200-400 copies/mL. However, of the 5 patients with the YMDD resistant mutant, only one had a high level viremia before starting TDF, while the other 4 had low viremia (1000-2000 copies/mL). Also, the 6 patients with wild-type virus were shifted to TDF when their PCR was negative. Thus, the majority of patients had negative or low level viremia at the start of TDF, and this can be explained because they were all on lamivudine treatment at the time of the shift. No washout period was allowed for the fear of inducing a hepatitis flare in patients with severe disease and cirrhosis. Without an off-treatment baseline viremia, we could not demonstrate that low-dose TDF was able to control HBV replication from the beginning, but it is noteworthy that TDF maintained PCR negativity for an extended period of time in 10 of 11 (90.9%) patients. In these patients, we did not observe any persistent viral rebound, with the exception of two transient low-level viral blips that disappeared spontaneously without changing the TDF dose. This confirms the efficacy of low-dose TDF in controlling HBV viremia and also the high barrier to resistance of this drug, even at doses as low as 75 mg.

To date there have been only 2 reports of TDF-associated mutations conferring resistance to the drug^[1,12]. This low level of resistance could be related to the fact that the great majority of the studies were on HIV-positive patients treated with other antiviral drugs, such as lamivudine and emtricitabine, in addition to TDF. Data on long-term TDF as monotherapy are scanty and this is probably the first report of an extended period of treatment with this drug at a dosage lower than 300 mg. It is also likely that a 75 mg dose of TDF is more potent than 10 mg of adefovir, and in fact 3 out of 7 patients had a persistent viral rebound when shifted from TDF to adefovir. This is in agreement with the findings of Van Bommel and Berg^[13] who observed a reactivation of viral replication after replacement of TDF 300 mg with adefovir 10 mg. Despite being equipotent *in vitro*, adefovir is thus much less potent *in vivo* than TDF and can not retain the TDF response, whether it has been achieved with a 300 mg or with a 75 mg dose of TDF. This suggests that the greater potency of TDF is not only related to a higher dosage, but also to intrinsic differences in the antiviral

effect of the two drugs, namely a different intracellular phosphorylation of TDF compared to adefovir^[14,15], or a longer intracellular half-life of the phosphorylated form^[16]. We can, therefore, conclude from our data that TDF at low dose can retain for a long period of time a full suppression of HBV viremia induced by the previous use of lamivudine.

This could have practical implications in two settings: the treatment of advanced stage hepatitis B in low-income countries and the treatment of cirrhotics with a borderline renal function. In the latter case, a low-dose nucleotide analogue could assure a good control of HBV viremia, while preserving renal function. The possibility of reducing the cost of therapy is also appealing for low-income countries, where the price of the other nucleos(t)ides, with the exception of lamivudine, would be prohibitive. Reducing the dose of TDF from 300 to 75 mg would bring the cost of one month of therapy down to US\$100, which is 30% less than lamivudine and one fifth the cost of adefovir or entecavir. Even combining low-dose TDF and lamivudine, the monthly cost of therapy would be less than half the cost of adefovir or entecavir monotherapy.

Chronic hepatitis B virus affects more than 400 million people worldwide, the majority of which are living in low-income countries. Despite the fact that current HBV therapy is too expensive for these countries, no specific guidelines have been published for the developing world. Lamivudine is cheap and its cost will be further reduced when it will be available as a generic drug. Its use, however, is hampered by the emergence of resistance^[4], and a report from Iran showed a good biochemical control in only half of the patients after one year of treatment^[17]. Cost-effectiveness analysis of alfa-interferon has shown contradictory results for health care systems with tight budgetary constraints^[18,19]. Lamivudine or adefovir monotherapy is not considered cost-effective^[19], while data are lacking on entecavir and telbivudine. Moreover, alfa-interferon therapy has an additional cost of syringes and a need of refrigerating the drug which may limit its use in the developing world. Sequential treatment has been proposed as a cost-effective strategy, but there are disagreements on which drugs should be used in sequence. Kanwal *et al*^[19] proposed lamivudine as the first drug with adefovir rescue for resistant cases, while Shepherd *et al*^[20] opted for alfa-interferon followed by lamivudine. There is thus no consensus about the most cost-effective and affordable therapeutic strategy for hepatitis B in the developing world and the use of a potent drug like TDF at a reasonable cost would be greatly helpful.

Our findings show that low-dose TDF can control HBV viremia, but there are several limitations in this study and further evaluation of low-dose TDF in a larger population is needed. First, all the patients were HBeAg-negative and our results can not be generalized to HBeAg-positive patients, which are usually highly viremic. It is worth considering, however, that HBeAg-negative disease is on the rise in the developing world, especially in Asian countries^[21], and that a higher viremia could be controlled by an initial period of full-dose TDF. Another limitation of our study is that analysis of viral dynamics during

TDF treatment has shown an important variability in viral decline among the treated patients, even when using a 300 mg dose^[22]. It seems therefore likely that the use of a low-dose in a larger sample may encounter a greater variability of response than that found in our small series. An additional problem is a practical one: TDF tablets are very difficult to divide into four parts and may require a crushing apparatus or good sight and technical skill by the patient. Last but not least it should be considered that in the developing world, HBV is often associated to HIV and that a low-dose TDF could more easily induce resistance to this drug and compromise first line therapy of HIV^[23]. It would therefore be advisable to use low-dose TDF only in advanced stage HBV disease when the prognosis of the underlying liver disease prevails over HIV infection, or alternatively to use TDF in association to low-cost antiretroviral therapy according to the World Health Organization Guidelines^[24].

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S- Editor Zhu LH L- Editor Kumar M E- Editor Yin DH

RAPID COMMUNICATION

Randomized, double-blind, comparative study of dexrabeprazole 10 mg *versus* rabeprazole 20 mg in the treatment of gastroesophageal reflux disease

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Received: 2007-04-18 Accepted: 2007-05-22

Abstract

AIM: To compare the efficacy and safety of dexrabeprazole 10 mg *versus* rabeprazole 20 mg in the treatment of gastroesophageal reflux disease (GERD).

METHODS: This was a randomized, double-blind clinical study. Fifty patients with GERD were randomly assigned to receive dexrabeprazole 10 mg or rabeprazole 20 mg once daily. Efficacy was assessed by evaluating improvement in visual analog scale (VAS) scores of heart-burn and regurgitation and safety was assessed by recording incidence of any adverse drug reactions. Laboratory investigations and upper gastro-intestinal endoscopy was conducted at baseline and after 28 d of therapy.

RESULTS: A total of 50 patients ($n = 25$ in dexrabeprazole group and rabeprazole group each) completed the study. There were no significant differences in the baseline characteristics between the two groups. The VAS score (mean \pm SD) of heartburn and regurgitation in dexrabeprazole (64.8 ± 5.1 and 64 ± 8.1 , respectively) and rabeprazole (64.4 ± 8.7 and 57.6 ± 9.7 , respectively) groups significantly reduced ($P < 0.0001$) to 30 ± 11.5 , 24 ± 10 and 32 ± 9.5 , 29.2 ± 11.9 , respectively on d 28. A significantly higher ($P = 0.002$) proportion of patients showed $\geq 50\%$ improvement in regurgitation with dexrabeprazole 10 mg (96%) compared to rabeprazole 20 mg (60%). Onset of symptom improvement was significantly earlier with dexrabeprazole than with rabeprazole (1.8 ± 0.8 d *vs* 2.6 ± 1.4 d; $P < 0.05$). The incidences of esophagitis in the dexrabeprazole group and rabeprazole group before therapy were 84% and 92%, respectively ($P = 0.38$). The incidence of improvement/healing of esophagitis after therapy was more ($P = 0.036$) in the dexrabeprazole group (95.2%) compared to the rabeprazole group (65.2%). No adverse drug reaction was seen in either group.

CONCLUSION: In the treatment of GERD, efficacy of dexrabeprazole 10 mg is better than rabeprazole 20 mg, with regards to improvement/healing of endoscopic lesions and relief from symptoms of regurgitation.

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Key words: Dexrabeprazole/R(+) rabeprazole; Gastroesophageal reflux disease; Efficacy; Safety

Pai V, Pai N. Randomized, double-blind, comparative study of dexrabeprazole 10 mg *versus* rabeprazole 20 mg in the treatment of gastroesophageal reflux disease. *World J Gastroenterol* 2007; 13(30): 4100-4102

<http://www.wjgnet.com/1007-9327/13/4100.asp>

INTRODUCTION

Rabeprazole is a mixture of two isomers: R(+) and S(-)^[1]. Efficacy of dexrabeprazole [R(+) rabeprazole] has been confirmed in animal studies at half the dose of the racemate with the R-isomer being more effective than S-isomer in aspirin-induced ulcers^[1]. Pharmacokinetics in human volunteers have shown that, irrespective of the metabolizer status, the ratio of R:S isomer of rabeprazole in terms of C-max was between 1.7 to 1.9, with the ratio for area under the curve (AUC) being between 1.8-2.4. This may explain why dexrabeprazole at lower dose than racemate can be as effective as racemate^[2].

MATERIALS AND METHODS

This was a randomized, double-blind, comparative, clinical study permitted by Drugs Controller General of India (DCGI) on November 29, 2006 and was conducted in compliance with the 'Guidelines for Clinical Trials on Pharmaceutical Products in India-GCP Guidelines' issued by the Central Drugs Standard Control Organization, Ministry of Health, Government of India (<http://www.cdsco.nic.in/html/GCP1.html>). The Ethical Committee Approval was taken from Independent Ethics Committee, Center for Behavioral Medicine, Pune, India. The study was initiated on December 5, 2006 and completed on April 5, 2007.

Male or female patients between 18-65 years of age,

Table 1 Results of endoscopic findings

Parameters	Baseline (d 0)		After therapy (d 28)		Analysis of findings				
	Dexrabeprazole 10 mg (<i>n</i> = 25)	Rabeprazole 20 mg (<i>n</i> = 25)	Dexrabeprazole 10 mg (<i>n</i> = 25)	Rabeprazole 20 mg (<i>n</i> = 25)	CER	TER	ARR	RRR	NNT
Patients with esophagitis ^[4]	21	23	8	15	0.65	0.38	0.27	42%	4
Grade A	1	7	8	11					
Grade B	20	16	0	4					
Grade C	0	0	0	0					
Grade D	0	0	0	0					
Investigator-reported improvement in endoscopic findings and healing									
-Yes	NA	NA	20	15	0.652	0.952	0.30	46%	3
-No	NA	NA	1	8					

NA: Not applicable; CER: Control event rate; TER: Test event rate; ARR: Absolute risk reduction; RRR: Relative risk reduction; NNT: Number needed to treat.

clinically diagnosed with GERD were included after obtaining written informed consent. Patients with a known history of hypersensitivity to any proton pump inhibitors (PPIs) or history of infectious or inflammatory conditions of the intestine such as inflammatory bowel disease, malabsorption syndromes, intestinal obstruction, gastrointestinal malignancy, gastric or intestinal surgery (vagotomy), Barrett's esophagus, esophageal stricture, pyloric stenosis, and scleroderma were excluded from the study. Pregnant and lactating females, patients with abnormal laboratory tests at baseline (including liver enzymes greater than twice the upper limit of normal), patients refractory to a 2-mo course of H₂-blocker or PPI therapy for GERD treatment, patients who took PPI within 14 d of screening or a H₂-blocker or prokinetic agent within 7 d of screening, patients who required daily use of non-steroidal anti-inflammatory drugs (NSAIDs), oral steroids, aspirin or who were unable to discontinue the use of anticholinergics, cholinergics, spasmolytics, opiates or sucralfate and patients with poorly controlled associated disease (such as heart disease, coagulation disorders, thyroid disorders) were also excluded from the study.

Enrolled patients were randomized (as per the computer generated randomization chart, www.randomization.com) in blocks of ten to receive identical looking tablets of dexrabeprazole 10 mg once daily (OD) or rabeprazole 20 mg OD in coated opaque envelopes to conceal the identity of the treatment allocated.

Patients recorded severity/relief of their symptoms on a visual analog scale (VAS)^[3] [at baseline (d 0) and on d 14 and 28 of therapy] and in a diary card (daily throughout the study duration). Laboratory investigations (SGPT, SGOT, serum creatinine, platelet count, total and differential WBC count) and upper gastro-intestinal endoscopy were conducted at baseline and after 28 d of therapy.

Statistical analysis

Student's *t* test was applied for VAS scores and Chi-square for proportions showing $\geq 50\%$ improvement. CLINSTAT software was used for statistical analysis (Martin Bland, CLINSTAT). *P* < 0.05 was considered statistically significant.

RESULTS

A total of 50 patients (*n* = 25, M:F = 16:9, mean age:

39.32 \pm 10.6 years, mean weight: 60.4 \pm 11.27 kg in 10 mg dexrabeprazole group; *n* = 25, M:F = 20:5, mean age: 35.7 \pm 6.4 years, mean weight: 64.3 \pm 13.4 kg in 20 mg rabeprazole group) completed the study. There were no significant differences in the baseline characteristics of the two groups. The VAS score (mean \pm SD) of heartburn and regurgitation in dexrabeprazole and rabeprazole groups reduced significantly (*P* < 0.0001) from 64.8 \pm 5.1, 64 \pm 8.1, and 64.4 \pm 8.7, 57.6 \pm 9.7 on d 0 to 42 \pm 10.4, 34.8 \pm 10.8 and 46.4 \pm 11.5, 35.4 \pm 10.8 on d 14 with further reduction to 30 \pm 11.5, 24 \pm 10 and 32 \pm 9.5, 29.2 \pm 11.9 on d 28 of the therapy, respectively. There was no significant intergroup difference in improvement of symptom scores. A significantly higher (*P* = 0.002) proportion of patients showed at least 50% improvement in symptoms of regurgitation with dexrabeprazole 10 mg (96%) than with rabeprazole 20 mg (60%). Onset of symptom improvement was earlier (*P* < 0.05) at 1.8 \pm 0.8 d with dexrabeprazole than with rabeprazole at 2.6 \pm 1.4 d. Endoscopy showed that the incidence of 'residual esophagitis' (any grade of esophagitis as per Los Angeles Classification^[4]) after 28 d was higher in the 20 mg rabeprazole group compared to 10 mg dexrabeprazole group. Similarly, incidence of healing was significantly higher (*P* = 0.036) in the dexrabeprazole group compared to the rabeprazole group (Table 1). This represents an absolute improvement of 30% and relative improvement of 46% over racemate. No adverse drug reaction was seen in either group. Laboratory parameters did not show any significant differences as compared to baseline.

DISCUSSION

GERD is characterized by recurrent return of gastric contents back into the esophagus. The goal of treatment is to improve patient's quality of life by providing rapid relief of symptoms and reducing the severity and number of recurrent episodes. Therefore, an important endpoint in clinical trials assessing the efficacy of treatment in GERD patients is time taken for complete relief of symptoms, especially the pivotal symptoms of heartburn and regurgitation. This can be measured as time to the first 24-h interval free from GERD symptoms of heartburn or acid regurgitation. Other endpoints include global symptom improvement, satisfactory, and complete relief of symptoms, *etc.*

In the present study, onset of action (time to the first 24-h interval free from GERD symptoms) was significantly earlier ($P < 0.05$) with dexrabeprazole than with rabeprazole. Although, the VAS score of heartburn and regurgitation in dexrabeprazole and rabeprazole groups reduced significantly ($P < 0.0001$), a higher ($P = 0.002$) proportion of patients showed at least 50% improvement in symptoms of regurgitation with 10 mg dexrabeprazole (96%) than with 20 mg rabeprazole (60%). Endoscopic findings also showed that the incidence of healing was significantly higher ($P = 0.036$) in the dexrabeprazole group as compared to the rabeprazole group (Table 1). This represents an absolute improvement of 30% and relative improvement of 46% over racemate, yielding an NNT (number of patients needed to treat to benefit at least one patient) of only 3 patients. No adverse drug reaction was seen in either group. Laboratory parameters did not show any significant differences as compared to baseline.

This study shows that in the symptomatic management of GERD, dexrabeprazole will provide better results than the racemate, even when used at half the normal dose. Our results are in conformity with previously published pre-clinical studies^[1], confirming the efficacy of dexrabeprazole at half the dose of the racemate. Thus it would, therefore, be advantageous to use the R(+) isomer in favor of the racemate to reduce metabolic load on the body, to simplify pharmacokinetics and have better efficacy with equal safety.

The therapeutic value of chiral purification of PPI racemates is already confirmed with earlier introductions of esomeprazole and S-pantoprazole^[5]. Findings of the present study strengthen this further.

In summary, for GERD treatment, efficacy of dexrabeprazole 10 mg is better than rabeprazole 20 mg, with regards to improvement and healing of endoscopic lesions and relief from symptoms of regurgitation.

ACKNOWLEDGMENTS

We thank Emcure Pharmaceuticals Ltd. for providing dexrabeprazole 10 mg and rabeprazole 20 mg tablets.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is very common, presenting as heartburn and regurgitation. GERD can be associated with inflammation of

esophageal mucosa (esophagitis) and even abnormal cellular changes (Barrett's esophagus). Proton pump inhibitors (PPIs) form the mainstay of therapy for GERD.

Research frontiers

PPIs are mixtures of enantiomers. Chiral purification of PPI is a useful way to improve existing therapy. Early improvement in healing of esophagitis is very important.

Innovations and breakthroughs

Chiral purification has previously allowed introduction of better version of omeprazole and pantoprazole as esomeprazole and S-pantoprazole in terms of better efficacy.

Applications

The existing racemate rabeprazole when used as chirally pure dexrabeprazole lessens the drug dosage, metabolic load on body, and provides faster healing of esophagitis with more patients getting relief from symptoms of GERD.

Terminology

GERD = Gastroesophageal reflux disease, VAS = Visual analog Scale used to score symptoms, CER = Control event rate (i.e. incidence with the reference product), TER = Test event rate (i.e. incidence with the test product), ARR = Absolute risk reduction (in the event of interest), NNT = number (of patients) needed to treat to benefit at least one patient.

Peer review

This is a randomized, double-blind clinical study comparing efficacy and safety of dexrabeprazole 10 mg versus rabeprazole 20 mg in the treatment of gastroesophageal reflux disease (GERD). The paper is well written and the results show efficacy of dexrabeprazole 10 mg is better than rabeprazole 20 mg, with regards to improvement/healing of endoscopic lesions and relief from symptoms of regurgitation

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S- Editor Zhu LH L- Editor Kumar M E- Editor Wang HF

Safety and efficacy of oral HD-03/ES given for six months in patients with chronic hepatitis B virus infection

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Received: 2006-10-13 Accepted: 2006-11-14

<http://www.wjgnet.com/1007-9327/13/4103.asp>

Abstract

AIM: To investigate the safety and efficacy of the formulation HD-03/ES capsules in the management of patients with chronic hepatitis B infection.

METHODS: A total of 25 patients were recruited to the study and were given HD-03/ES, two capsules twice daily for six months. Clinical assessment of symptoms and signs were done using the "clinical observation table" once a month before and after the treatment. Biochemical investigations of total bilirubin, ALT, AST, serum protein for liver function tests were done every month after initiating treatment. Serum was analyzed for HBV markers for HBsAg, HBeAg and HBV DNA at baseline, 4 and 6 mo after therapy using ELISA kits from Roche.

RESULTS: After 6 mo of therapy with HD-03/ES, a significant reduction of ALT values from 66.5 ± 11.1 to 39.1 ± 5.2 ($P < 0.01$) and a significant HBsAg loss (52%, $P < 0.001$), HBeAg loss (60%, $P < 0.05$) and HBV DNA loss (60%, $P < 0.05$) was observed. Adverse effects were mild and never warranted withdrawal of the drug.

CONCLUSION: The results of this pilot study indicate that HD-03/ES might be a safe and effective treatment for chronic hepatitis B infection and a long-term multicentric comparator trial is warranted and under way.

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Key words: HD-03/ES; Chronic hepatitis B; Liver function tests; Hepatitis B Virus markers; Clinical trial; HBsAg; HBeAg

Rajkumar JS, Sekar MG, Mitra SK. Safety and efficacy of oral HD-03/ES given for six months in patients with chronic hepatitis B virus infection. *World J Gastroenterol* 2007; 13(30): 4103-4107

INTRODUCTION

Hepatitis B virus (HBV) is a hepadnavirus that is noncytopathic and causes significant morbidity and mortality worldwide^[1]. Chronic hepatitis B (CHB) affects an estimated 400 million people worldwide with over 50 000 fatalities each year^[2]. 82% of the world's 530 000 cases of liver cancer per year are caused by viral hepatitis infection, with 316 000 cases associated with hepatitis B infection^[3]. According to a WHO report, India has intermediate endemicity of hepatitis B, with Hepatitis B surface antigen (HBsAg) prevalence between 2% and 7% among populations studied. The prevalence does not vary significantly by region in the country. The number of HBsAg carriers in India has been estimated to be over 40 million (4 crore). It has been estimated that, in India, of the 25 million infants born every year, over one million run the lifetime risk of developing chronic HBV infection. Every year over 100 000 Indians die due to illnesses related to HBV infection.

Ayurveda, an indigenous system of medicine in India, has a long tradition of treating liver disorders with plant drugs^[4]. On the basis of leads available from folklore usage and recent experimental studies, HD-03/ES (a capsule formulation consisting of 125 mg each of hydroalcoholic extracts of the herbs *Cyperus rotundus* and *Cyperus scariosus*) was evolved to elicit hepatoprotective activity.

Surface antigen suppression and HBV elimination activities of herbal extract containing *Cyperus rotundus* and *Cyperus scariosus* were examined using two HBsAg expressing human hepatocellular carcinoma cell lines, PLC/PRF/5 and HepG2.2.215 polymerase chain reaction (PCR) for the study of amplification of DNA specific to HBV, reverse transcriptase inhibition assay, immunomodulatory effects and hepatoprotective ability against oxidative damage to hepatocytes were some of the other studies performed to evaluate the efficacy of the plant extract. The efficacy of the plant extract to eliminate the duck hepatitis B virus was assessed in experimentally infected Pekin ducks in a duck model study. Our investigations indicated that the extracts could reversibly inhibit cell growth and suppress HBsAg expression in both of the human hepatocellular carcinoma cell line models. Acute and sub-acute toxicity studies conducted in rats indicated that HD-03/ES is devoid of significant toxicity

following acute and repeated administration in rats (Data on file).

A preliminary case study report indicated that there was significant reduction of HBsAg along with disappearance of viral DNA in a patient treated with HD-03/ES at a dosage of two capsules twice daily for a period of six months^[5]. At the moment, there is no data to show whether HD-03/ES treatment is adequate for the treatment of HBV infection. We therefore, undertook this clinical study to evaluate the safety and efficacy of HD-03/ES in patients with chronic hepatitis B infection.

MATERIALS AND METHODS

Patients

An open prospective controlled clinical trial was carried out in the Department of Gastroenterology, Lifeline Rigid Hospitals, Chennai, Tamilnadu, India, between March 2005 and June 2006 to evaluate the safety and efficacy of HD-03/ES capsules alone in the management of chronic hepatitis B infection. Informed written consent was obtained from all study participants and the protocol of the study was approved by the ethical committee of the institute. The study in general was conducted in accordance with Declaration of Helsinki and GCP Guidelines issued by the Ministry of Health, Government of India.

Diagnostic criteria

Patients with a history of hepatitis B or HBsAg carriers for at least 6 mo, who still had symptoms and signs of hepatitis as well as abnormal liver function and positive HBsAg, were diagnosed as having CHB infection in the present study.

Criteria for enrollment

Patients, aged 18-60 years, with their serum alanine aminotransferase (ALT) level being 41-200 IU/L and who had positive serum HBsAg, were enrolled.

Criteria for exclusion

Patients aged over 60 years or less than 18 years, pregnant or lactating women, patients who had hepatitis C or other hepatic viral infection, autoimmune hepatitis and drug-induced hepatitis or alcoholic hepatitis; patients with severe complications of the cardiovascular, renal or hematopoietic system; and patients with mental diseases, were excluded. Patients were excluded if they had decompensated liver disease (defined by serum albumin \leq 360 g/L, bilirubin \geq 150 g/L, prothrombin time \geq 2 s prolonged, or a history of ascites, variceal hemorrhage or hepatic encephalopathy), pancytopenia (defined as hemoglobin $<$ 110 g/L, white cell count $<$ 4000/mm³ or platelets $<$ 105/mm³). Patients with a history of using interferon or anti-viral agents or corticosteroids or immunosuppressive drugs were also excluded.

Treatment

Each patient was asked to take two capsules of HD-03/ES (The Himalaya Drug Company, Bangalore, India) twice daily, two capsules in the morning and two capsules at bedtime after food for a period of six months.

Recording and observation of symptoms and signs

The symptoms and signs of patients were recorded in detail using the "Clinical Observation Table" once a month before and during the treatment.

Etiological markers of hepatitis B

Serum samples collected from patients were stored at -20°C until analysis. Serum was assayed for HBsAg, hepatitis B e-Antigen (HBeAg), and HBV DNA at baseline, four and six months after therapy using commercially available enzyme-linked immunosorbent assay kits from Roche.

Liver function

The patients had liver function examinations every month during the treatment, including contents of serum proteins, total bilirubin (TB) and activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Safety analysis

Safety analysis included data for all treated patients during dosing. The primary safety end point was discontinuation of study medication because of adverse events. Other safety evaluations included incidence of adverse effects.

End points

The primary end point was HBsAg clearance. Secondary endpoints included HBV DNA levels and ALT normalization to 40 IU/L at the end of treatment.

Statistical analysis

The intention-to-treat analysis included all randomized patients who were HBsAg-positive at baseline and received at least one dose of the study medication. Data were expressed as mean \pm SD. One-way ANOVA with Bonferroni's multiple comparison test or Dunnett's multiple comparison test was performed wherever appropriate using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA. A *P* value of $<$ 0.05 was taken as statistically significant.

RESULTS

Twenty five patients (22 males and 3 females) aged between 20 and 45 years with a mean age of 33.7 years participated in this open study. Their baseline characteristics are shown in Table 1.

Clinical response

Six months of therapy with HD-03/ES capsules was markedly effective in the majority of the patients as it resulted in disappearance or alleviation of chief clinical symptoms such as abdominal pain and poor appetite. The effect of six months of treatment with HD-03/ES on liver function tests are shown in Table 2. As shown in the table, there was a trend towards normalization of liver function tests in all patients treated with HD-03/ES.

ALT normalization

Levels of ALT before and after six months of treatment with HD-03/ES are shown in Table 3. After six months

Table 1 Demographic data

Characteristic		HD-03/ES
Age (yr)	Mean (SD)	33.7 (6.6)
	Median (range)	36 (20-45)
Sex	Male	22
	Female	3
Body weight (kg)	Mean (SD)	56 (10)

Table 2 Effect of six months treatment with HD-03/ES on liver function tests (mean \pm SD)

Parameter	d 0	Four months	Six months
Alanine aminotransferase (ALT) (IU/L)	66.5 \pm 11.1	41.6 \pm 05.1	39.1 \pm 5.2
Aspartate aminotransferase (AST) (IU/L)	47.5 \pm 9.5	42.4 \pm 10.7	40.2 \pm 10.1
Serum albumin (g%)	3.5 \pm 0.8	3.5 \pm 0.7	3.6 \pm 0.7
Serum globulin (g%)	2.9 \pm 0.3	3.1 \pm 0.2	3.2 \pm 0.2
Total protein (g%)	6.2 \pm 0.7	6.5 \pm 0.7	6.5 \pm 0.7
Serum bilirubin (mg%)	1.3 \pm 0.6	1.2 \pm 0.5	1.1 \pm 0.5
Alkaline phosphatase (IU/L)	155.5 \pm 9.8	140.2 \pm 9.0	127.0 \pm 7.5

Table 3 Biochemical and serological response to 6 mo of HD-03/ES therapy

Variable		HD-03/ES (No. of patients)	
ALT normalization (%)	Four months	32%	
	Six months	56%	
HBsAg		Positive	Negative
	d 0	25	0
	Four months	19	6
HBeAg	Six months	12	13 ^b
	d 0	16	9
	Four months	11	14
HBV DNA	Six months	10	15 ^a
	d 0	16	9
	Four months	11	14
	Six months	10	15 ^a

^a $P < 0.05$; vs d 0; ^b $P < 0.001$ vs d 0.

of treatment, the levels of ALT were decreased from initial value of 66.5 ± 11.1 to 39.1 ± 5.2 , and this reached levels of statistical significance ($P < 0.01$). In 14 of the 25 patients (56%), ALT levels were normalized. Although ALT levels were not normalized in the remaining 11 patients, there was a trend towards reduction and in none of the patients was a rise in ALT levels seen (Figure 1).

Virological response

The effect of 6 mo of treatment with HD-03/ES treatment on virological response is shown in Table 3. Thirteen of the 25 patients (52%) at the end of treatment, who were treated with HD-03/ES, had undetectable HBsAg. This difference was statistically significant ($P < 0.001$) (Figure 2). HBeAg loss (60%, $P < 0.001$) and HBV DNA loss (60%, $P < 0.05$) also occurred during treatment with HD-03/ES in that 6 patients who were positive for both HBeAg and HBV DNA initially, but were negative for the same at the end of therapy (Figure 3 and Figure 4).

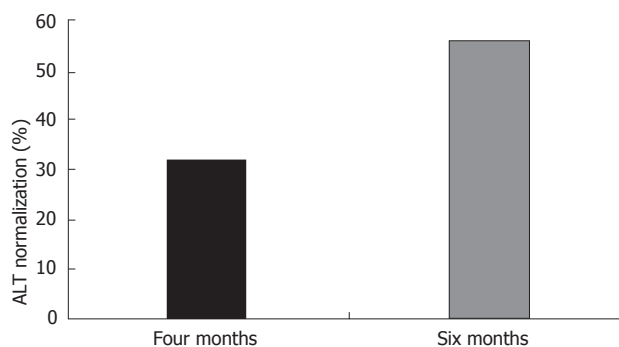
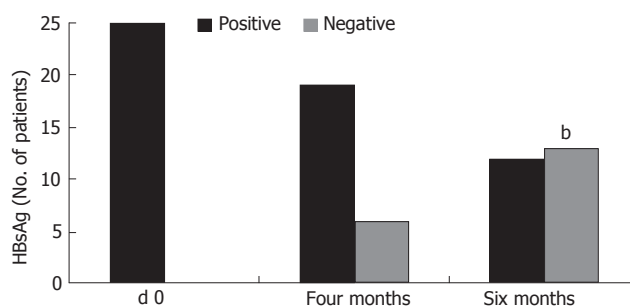
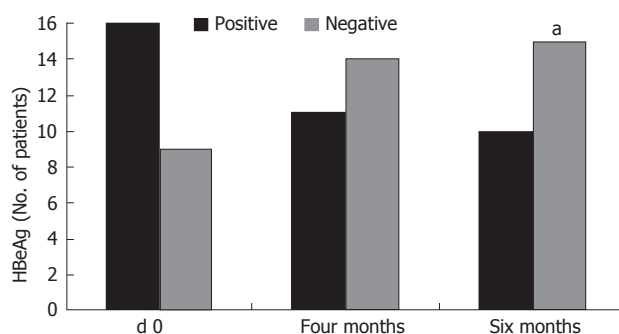


Figure 1 Improvement in ALT normalization after treatment with HD-03/ES.

Figure 2 Improvement in HBsAg after treatment with HD-03/ES. ^b $P < 0.001$ vs d 0.Figure 3 Improvement in HBeAg after treatment with HD-03/ES. ^a $P < 0.05$ vs d 0.

Adverse events

HD-03/ES was well tolerated in this study. No patient was withdrawn from therapy either for adverse effects or for other reasons. The adverse events observed during therapy are shown in Table 4. Most of the observed side-effects were mild (fatigue, headache and insomnia) in nature. The most common adverse event was abdominal discomfort. No serious biochemical abnormalities were experienced by any patient.

DISCUSSION

High morbidity and mortality have been found in Asia among HBsAg-positive patients, even in the absence of overt liver disease^[6,7]. The goals of treatment in CHB infection are sustained viral suppression, normalization of ALT levels and improvement in liver histology leading to long-term reduction in the risk of cirrhosis and hepatocellular carcinoma^[8]. Loss of HbsAg, HBeAg

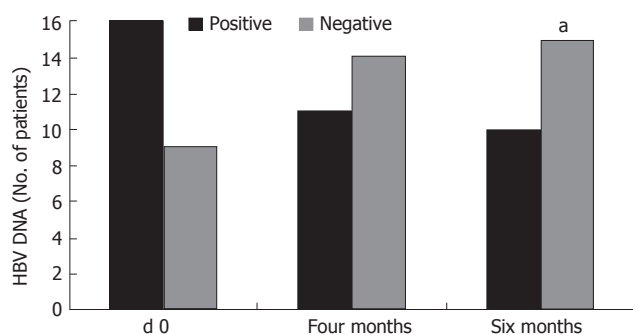


Figure 4 Improvement in HBV DNA after treatment with HD-03/ES.^a $P < 0.05$ vs d 0

and normalization of ALT levels and improvement in liver histology are the usual short-term end points of therapy^[9]. The results of this preliminary study indicate that short-term therapy with HD-03/ES is effective in the management of CHB. Although the initial results of this study are promising, it remains to be seen whether virological response will be sustained during chronic dosing and whether relapse rates after cessation of therapy would be low unlike conventional therapies whose relapse rates are high after treatment cessation^[10].

The ultimate endpoint of antiviral therapy for CHB infection is loss of HBsAg, which is accompanied by disease remission in terms of ALT normalization^[11]. In this study, HBsAg loss was observed in 52% of the patients after 6 mo of therapy with HD-03/ES. This is in contrast to several clinical trials of lamivudine or adefovir where HBsAg loss was not reported^[12,13] or tends to occur later than 24 wk as with interferon therapy^[14]. Although six months of therapy is limited and not capable of inducing pronounced viral suppression, five patients lost their HBV DNA after six months of therapy, which is highly encouraging.

Loss of HBeAg either spontaneously or following therapy significantly improves the clinical outcome and survival in chronic HBV patients. Therefore, HBeAg loss has remained as a major end point of antiviral therapy in chronic HBV infection. Monotherapy with alpha interferon for 16 to 26 wk is associated with loss of serum HBeAg in 20%-40% of the patients^[15]. Our results (55%) are slightly better.

The possible mechanisms of action as studied using HBsAg expressing human hepatocellular carcinoma cell lines PLC/PRF/5 and HepG2.2.2.15 indicate to HBsAg suppression by binding to the antigen, and HBV elimination by reverse transcriptase inhibition. Immunomodulatory effects occur by causing the release of nitric oxide (NO) by macrophages and cytokines like TNF- α . It was found to have a hepatoprotective effect by reversing the oxidative damage caused by hepatocytes.

A strong correlation was found between HBV DNA levels and histology activity index scores in HBeAg negative patients^[16]. As ALT levels are consistent with histological activity index scores, the findings in the present study of ALT normalization, HBsAg loss together with loss of DNA during short-term treatment with HD-03/ES indicates that patients treated with HD-03/ES may lose their infectivity faster and relapse rates would be low.

Table 4 List of adverse effects

Adverse effect	HD-03/ES
Abdominal discomfort	3
Fatigue	2
Headache	1
Insomnia	1

Although the initial results of this study are promising, it remains to be seen whether virological response will be sustained during chronic dosing and whether relapse rates after cessation of therapy would be low unlike conventional therapies whose relapse rates are high after treatment cessation^[17]. Our study has several obvious limitations and among these we should consider the small sample size.

In summary, this trial demonstrated that 24 wk of HD-03/ES treatment resulted in clinically significant virological and biochemical benefits in patients with CHB infection. Hence to conclude the potential benefit of HD-03/ES in the management of CHB, HD-03/ES should be studied in long-term comparative trials with standard drugs with extended duration of follow-up.

ACKNOWLEDGMENTS

We thank all the trial participants for their consent and participation in this study. We thank the management Lifeline Rigid Hospitals, Kilpauk, Chennai-600010, India, for kind permission to conduct the study and publish the results.

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S- Editor Liu Y L- Editor Alpini GD E- Editor Wang HF



RAPID COMMUNICATION

Detection and treatment of synchronous lesions in colorectal cancer: The clinical implication of perioperative colonoscopy

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Received: 2007-05-08 Accepted: 2007-05-12

Key words: Colon cancer; Synchronous colon polyp; Intraoperative colonoscopy

Kim MS, Park YJ. Detection and treatment of synchronous lesions in colorectal cancer: The clinical implication of perioperative colonoscopy. *World J Gastroenterol* 2007; 13(30): 4108-4111

<http://www.wjgnet.com/1007-9327/13/4108.asp>

Abstract

AIM: To evaluate the clinical significance of pre- and intra-operative colonoscopy for the detection of synchronous lesions in colon cancer.

METHODS: Two hundred and sixty-five pre-operative and 51 intra-operative colonoscopic evaluations were performed in 316 colorectal cancer patients who underwent curative resection from January 2001 to June 2006. The incidence and characteristics of synchronous lesions and their influence on surgery were evaluated.

RESULTS: Two hundred and eighty-two synchronous lesions were detected in 124 (39.2%) of 316 patients including all lesions regardless of their histologic type. True adenomatous polyps were found in 91 (28.8%) of 316 patients, and 17 (5.4% of all patients) patients had synchronous colon cancers. The preoperative identification of synchronous lesions altered the planned surgery in 37 (14.0%) of 265 patients. In 18 patients among the surgically removed cases, the lesions were removed by extending the resection range. Further segmental resection or polypectomy through enterotomy was necessary in 19 patients. Nineteen (37.2%) of 51 intraoperative colonoscopy cases had synchronous lesions. Additional surgical procedures including segmental bowel resection and polypectomy with enterotomy were necessary in 7 (13.7%) of 51 intraoperative colonoscopy cases to remove the lesions.

CONCLUSION: Synchronous colorectal polyps or cancer are frequent and their preoperative detection is important for optimal surgical planning and treatment. Intraoperative colonoscopy is a useful option in cases where a preoperative colonoscopy is not feasible.

INTRODUCTION

The association of synchronous adenomatous polyps in colon cancer has been reported to be 15%-50% and synchronous cancer as high as 2%-10%^[1-4]. A routine preoperative colonoscopy has been recommended for patients diagnosed with colorectal cancer in order to identify synchronous polyps and/or cancer, that otherwise might have remained undetected at the time of the surgery^[5]. The identified lesions can be removed endoscopically or by surgery.

Despite its importance, a preoperative colonoscopy of the entire colon is often unobtainable due to bowel obstruction by the tumor, poor bowel preparation or limitations associated with available facilities. Several authors have shown the usefulness of intraoperative colonoscopy when a preoperative colonoscopy was not possible^[6,7]. The detection of synchronous tumors by intraoperative colonoscopy often alters the planned surgery. However, not all investigators agree on the effectiveness of intraoperative colonoscopy. Among the concerns reported are the increased surgical time and possible risk of infection^[8].

The prevalence of colon cancer and polyps differ widely by race and geographic location. The clinical significance of synchronous polyps or cancer may be different based on these epidemiologic factors. Most prior studies on perioperative colonoscopy have been performed in Western countries; there are only a limited number of reports in Asia^[9,10]. The present study was performed to evaluate the status of synchronous colon cancer in Korea and efficacy of perioperative colonoscopy.

MATERIALS AND METHODS

From January 2001 to June 2006, 324 consecutive surgeries for colorectal cancer, with intent-to-cure, were performed

Table 1 Distribution of primary carcinoma by stage (TNM stage) and site

Site	Stage				Total
	I	II	III	IV	
Ascending colon	16	22	16	3	57
Transverse colon	5	6	8	2	21
Descending colon	3	6	5	1	15
Sigmoid colon	22	27	43	3	95
Rectum	32	37	54	5	128
Total	78	98	126	14	316

at the Ilsanpaik Hospital.

A complete preoperative colonoscopy is a standard part of patient evaluation prior to surgery at this hospital. Intraoperative colonoscopy was performed when a preoperative colonoscopy was impossible or incomplete. The most common cause of failure of a preoperative colonoscopy was luminal narrowing by a tumor. The use of a bowel stent (Wallstent™, Boston Scientific, USA) often enabled preoperative bowel preparation in obstructed cancer and made elective surgery possible. Patients were placed in the lithotomy position if an intraoperative colonoscopy was anticipated. The surgeon performed the colonoscopy. Intra-abdominal hand guidance by an assistant surgeon made scope insertion easy. A noncrushing bowel clamp was applied at the terminal ileum to prevent gaseous distention of the small bowel, which is frequently encountered during the procedure. The intraoperative colonoscopy was performed after on-table bowel cleansing, when a preoperative stent insertion was impossible or an emergency operation was necessary. Intraoperative bowel cleansing was achieved with saline infusion through a Foley catheter inserted through the appendiceal stump as previously reported^[11]. The solid fecal residue was irrigated from the proximal colon and drained through an anesthetic scavenger tube that was connected to the distal colon. The colonoscopy was performed just after bowel anastomosis. Fifty-one intraoperative colonoscopy examinations were performed, and the synchronous lesions detected were removed by colonoscopic polypectomy or by a surgical approach depending on the size and features of the lesion.

Out of the 324 consecutive patients, 8 patients whose first colonoscopy evaluation was done postoperatively were excluded and the data on the remaining 316 patients (202 males and 114 females, mean age 61.5 ± 12.1 years) were analyzed. The location and pathologic stage of primary cancers in these 316 patients are given in Table 1. All the procedures, particularly the intraoperative bowel cleansing and colonoscopy, were performed with the written consent of the patients or their guardians.

RESULTS

Characteristics of synchronous lesions

Two hundred and eighty-two synchronous lesions, including polyps and carcinoma, were detected in 124 (39.2%) of 316 patients enrolled in the present study. All lesions, regardless of their histological type, were included.

Table 2 Characteristics of synchronous lesions

Characteristics	Number of lesions	%
Location of polyps		
Ascending colon	48	17.0
Transverse colon	39	13.8
Descending colon	18	6.4
Sigmoid colon	93	33.0
Rectum	84	29.8
Size of polyp (mm)		
< 5	77	27.3
5-10	109	38.7
10-15	56	19.8
15-20	23	8.2
> 20	17	6.0
Histology		
Carcinoma	19	6.7
Adenomatous polyp	158	56.0
Tubular	93	
Villo-tubular	37	
Villous	28	
Hyperplastic polyp	78	27.7
Others	27	9.6
Total number of polyps	282	100

Multiple lesions were common. Forty-four patients had only one lesion but 48 patients had two or three, 28 patients had 4-10, and four patients had more than 10 lesions. The average number of lesions per patient was 2.3. Adenomatous polyps were the most frequent histological type and hyperplastic polyps were the second most common. True adenomatous polyps were present in 91 (28.8%) of 316 patients (Table 2). Nineteen synchronous colon cancers were detected in 17 (5.4%) patients in the study population. The invasion depth of the synchronous cancer was mostly confined to the mucosa or submucosa. Fifteen tumors were mucosal or submucosal tumors, three tumors invaded muscle and four tumors invaded beyond the muscle layer.

Treatment of synchronous lesions detected at preoperative colonoscopy

Synchronous polyps were detected in 105 (39.6%) of 265 patients who underwent preoperative colonoscopy. Removal of the polyps detected at the preoperative colonoscopy was attempted by endoscopic modalities where technically feasible. Surgical removal was preferred if the polyp was close to the primary tumor or if the endoscopic removal was technically difficult. Another indication for surgical removal was an unfavorable histopathologic finding of the endoscopic polypectomized specimen.

One hundred and fifty-six lesions were removed from 77 patients by preoperative colonoscopic polypectomies. Ninety-nine lesions from 37 patients were surgically removed. Among them, 51 polyps in 18 patients were included in the surgical specimen. An extended resection was often necessary for the inclusion of polyps within specimen. A tattooing with indigocanine during the colonoscopy was helpful for determining the extent of the resection. Fifteen polyps in 10 patients were removed by polypectomy through enterotomy. An additional bowel

Table 3 Treatment of synchronous lesions

	No. of patients with synchronous lesions	No. of lesions
Preoperative colonoscopy cases (<i>n</i> = 265)	105 ¹	255
Preoperative polypectomy	77	156
Surgical removal	37	99
Included within specimen	18	51
Segmental resection	6	12
Enterotomy/wedge resection	10	15
Subtotal colectomy	3	21
Intraoperative colonoscopy cases (<i>n</i> = 51)	19	33
Endoscopic polypectomy	5	7
Surgical removal	7	13
Segmental resection	3	7
Enterotomy	4	6
Included within specimen ²	7	13
Total	124	282

¹Dual procedure were performed for some patients, therefore, the sum exceeds the total number of patients. ²These lesions were not recognized until postoperative specimen examination.

resection was necessary in six cases and subtotal colectomy was performed in three cases (Table 3).

Treatment of synchronous lesions detected at intraoperative colonoscopy

Fifty-one intraoperative colonoscopies were performed. The most frequent indication was the evaluation of the remainder of the colon when a segmental resection was performed in obstructed left side colon cancers. Out of 36 obstructed left colon cancer patients, preoperative bowel preparation was possible in 15 patients without the aid of a stent although the colon lumen was so narrowed that the colonoscope could not pass the lesion site. A colon stent was inserted in 12 of 36 patients for bowel cleansing. Intraoperative colonoscopy was performed after bowel anastomosis. Colonoscopy was performed after on-table colon lavage and bowel anastomosis in nine patients whose bowel preparation was not previously possible at all.

Preoperative colonoscopies were not done or were incomplete in nine patients who had causes other than bowel stenosis. These nine patients and six other patients whose lesion site was unclear at laparotomy accounted for additional cases that needed intraoperative colonoscopy. An average of 10.5 min was required for the intraoperative colonoscopy except when a polypectomy was performed.

Twenty synchronous cancers or clinically significant polyps were present in 12 (23.5%) of 51 patients who underwent intraoperative colonoscopy. Seven lesions from five patients were removed using an endoscopic snare. Three patients underwent further segmental colon resection and the polyp was removed through enterotomy in four patients. The seven resected specimens included 13 more lesions, which were not recognized until postoperative specimen examination. The results of this study showed that intraoperative colonoscopy does not significantly increase complication rates although a statistical comparison was impossible due to the limited case number (Table 4).

Table 4 Incidence of complications

Time of colonoscopies	Number of patients (%)
Preoperative colonoscopy (<i>n</i> = 265)	
Anastomosis leakage	4 (1.5)
Intraabdominal sepsis	3 (1.1)
Wound infection	7 (2.6)
Intraoperative colonoscopy with bowel preparation (<i>n</i> = 42)	
Anastomosis leakage	1 (2.4)
Intraabdominal sepsis	1 (2.4)
Wound infection	3 (4.7)
Intraoperative colonoscopy with intraoperative colon lavage (<i>n</i> = 9)	
Anastomosis leakage	0
Intraabdominal sepsis	0
Wound infection	2

DISCUSSION

The prognosis of colon cancer has improved with advances in early detection. However, a large number of patients with colorectal cancer still die from the recurrent disease after surgery^[12]. Overlooked synchronous lesions may adversely affect patient prognosis although local recurrence and distant metastases are the most common causes of treatment failure.

The results of this study showed the usefulness of pre- and intra-operative colonoscopy. The incidence of observed synchronous adenomatous polyps (28.8%) and carcinoma (5.4%) was consistent with previous studies^[3,4]. Ninety-four lesions were neoplastic tumors greater than 10 mm and thus of significant malignant potential. These findings suggest that a colonoscopy is essential at surgery in patients with colon cancer.

A preoperative colonoscopy is ideal in that it allows for surgical planning for the synchronous lesions when present. However, a preoperative colonoscopy is often impossible due to bowel obstruction. Given the necessity of a perioperative colonoscopy, the consequence of an unsuccessful preoperative procedure is to repeat the examination soon after operation. In order to avoid multiple invasive and expensive investigations, Barlow *et al*^[13], proposed performing colonoscopy only after surgery when the colonoscopy is more likely to be successful, with a reported failure rate of 10%. However, this policy may increase the risk for additional surgeries.

In our experience, modification of the standard resection was required in 25 of 105 patients who had polyps detected at the preoperative colonoscopy. Considering the lack of sensitivity of operative palpation, it is possible that some of these 25 patients would have required reoperation if the preoperative colonoscopy had not been performed. The avoidance of additional operations is a powerful argument for a preoperative colonoscopy. In our view, a preoperative colonoscopy should be routinely done whenever possible. After the colonoscopy and curative resection, the follow-up would begin with a patient assumed to have a clean colon.

It has been reported that a preoperative colonoscopy was not possible in as many as 50% of cases although our study shows a lower rate^[13,14]. Tumor stenosis of the

colon was the most frequent cause of not being able to do a preoperative colonoscopy. Considering the importance of precise information on synchronous lesions at colon cancer surgery, intraoperative colonoscopy is an attractive option. Preoperative bowel preparation was possible with or without the aid of a bowel stent in 27 of 36 cases of obstructed colon cancer patients in this study. An intraoperative colonoscopy could be done without much difficulty in a cleaned colon with the limited additional labor needed for preparation of the endoscopic device. The procedure added only 10 min of operation time, but the results were significant. We encountered synchronous lesions in 19 (37.2%) of 51 intraoperative colonoscopy cases and performed appropriate treatment for each including seven surgical removals.

The insertion of a stenting device is not always possible despite its usefulness in obstructed left side colon cancer. There are several surgical options when all of the trials for preoperative bowel preparation fail. The traditional treatment is a two-stage Hartmann procedure^[15]. Single stage procedures have also been tried with reports of positive results in most cases^[11,16-17]. A subtotal colectomy and the standard bowel resection, after an on-table bowel preparation with antegrade colonic irrigation, are the two most popular procedures. There are claims that primary resection with on-table bowel preparation is a better approach in terms of risk and cost compared to the two-stage procedure, and is superior to the subtotal colectomy in the long-term functional outcome^[18]. However, the procedure has the disadvantage that the surgeon does not know the status of synchronous lesions in addition to the risk of wound contamination. In our experience, intraoperative colonoscopy effectively solved this problem. Colonoscopy was possible in all cases with intraoperative bowel preparation although the case number was limited. Recently introduced devices for colonic irrigation may facilitate the procedure although we have no experience with them^[19].

There is a report that follow-up colonoscopy detects metachronous cancer in the first 2 to 3 postoperative years^[13]. However, two types of metachronous cancer must be distinguished as suggested by Heald *et al*^[20]. The lesion detected in the early postoperative period may be an overlooked synchronous lesion and thus should be differentiated from a genuine metachronous neoplasm found later. The reported mean time for a real metachronous cancer to develop is about 10 years. Therefore, once the entire colon has been inspected and all polyps found have been removed, a routine colonoscopy is not expected to detect metachronous cancers in the early postoperative years.

In conclusion, a preoperative colonoscopy should be performed for all colorectal cancer patients since the presence of synchronous lesions often alters the treatment plan. Intraoperative colonoscopy is a valid option when the preoperative evaluation is not possible due to bowel obstruction.

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RAPID COMMUNICATION

Duodeno-jejunal junction dyssynergia: Description of a novel syndrome

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Received: 2007-05-09 Accepted: 2007-06-09

Abstract

AIM: To investigate the hypothesis that duodeno-jejunal dyssynergia existed at the duodeno-jejunal junction.

METHODS: Of 112 patients who complained of epigastric distension and discomfort after meals, we encountered nine patients in whom the duodeno-jejunal junction did not open on duodenal contraction. Seven healthy volunteers were included in the study. A condom which was inserted into the 1st duodenum was filled up to 10 mL with saline in increments of 2 mL and pressure response to duodenal distension was recorded from the duodenum, duodeno-jejunal junction and the jejunum.

RESULTS: In healthy volunteers, duodenal distension with 2 and 4 mL did not produce pressure changes, while 6 and up to 10 mL distension effected significant duodenal pressure increase, duodeno-jejunal junction pressure decrease but no jejunal pressure change. In patients, resting pressure and duodeno-jejunal junction and jejunal pressure response to 2 and 4 mL duodenal distension were similar to those of healthy volunteers. Six and up to 10 mL 1st duodenal distension produced significant duodenal and duodeno-jejunal junction pressure increase and no jejunal pressure change.

CONCLUSION: Duodeno-jejunal junction failed to open on duodenal contraction, a condition we call 'duodeno-jejunal junction dyssynergia syndrome' which probably leads to stagnation of chyme in the duodenum and explains patients' manifestations.

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Key words: Epigastric distension; Vomiting; Nausea; Dyspepsia; Chyme

Shafik A, Shafik IA, El Sibai O, Shafik AA. Duodeno-jejunal

junction dyssynergia: Description of a novel syndrome. *World J Gastroenterol* 2007; 13(30): 4112-4116

<http://www.wjgnet.com/1007-9327/13/4112.asp>

INTRODUCTION

The stomach passes chyme to the duodenum (DD) in jets under the control of physiologic antropyloric mechanisms^[1-3]. In addition to the chyme, the DD contents comprise mucosal secretions of the DD mucosa, pancreatic secretions such as pancreatic enzymes, lysolecithin and bicarbonate as well as bile as a composition of bile acids, pigments and bilirubin^[4-6]. These DD contents are passed to the jejunum (JJ). During their passage through the DD, they are claimed to be controlled by DD sphincters. In addition to the gastroduodenal pyloric sphincter, other controversial sphincters have been described to exist in the DD^[7-9]. One sphincter is alleged to be located at the distal end of the DD bulb and may be responsible for segmental achalasia and megabulb^[7]. Another sphincter is said to exist proximal to the ampulla of Vater^[8]. The 'Ochsner muscle' was described by Ochsner to be found below the ampulla of Vater^[9]. All of these sphincters were suggested to delay the passage of chyme through the DD so as to become thoroughly mixed with the biliary and pancreatic secretions. However, these sphincters have as yet not been verified as anatomical entities^[10].

A previous study had demonstrated a high- pressure zone at the duodenojejunal junction (DJJ), denoting that the DJJ might act as a physiological sphincter^[11]. The DJJ pressure had been demonstrated to diminish on duodenal contraction and increase on jejunal contraction^[11]. This effect was suggested to be reflex and mediated through the 'duodeno-jejunal junction inhibitory-' and the 'duodeno-jejunal junction excitatory-' reflexes^[11]. The former seems to allow the chyme to pass to the JJ from the DD, and the latter reflex upon jejunal contraction appears to prevent reflux of jejunal contents into the DD.

During our study of the duodenal motile activity of 112 patients who complained of epigastric distension and discomfort after meals, we encountered 9 patients in whom the DJJ did not open on DD contraction. We hypothesized the existence of duodeno-jejunal dyssynergia at the DJJ in these patients. This hypothesis was investigated in the current study.

Table 1 Effect of duodenal distension on the pressure in the duodenum (DD), duodeno-jejunal junction (DJJ), and jejunum (JJ) in the healthy volunteers and patients (mean \pm SD)

Distension volume (mL)	Pressure (cm H ₂ O)											
	Volunteers						Patients					
	DD		DJJ		JJ		DD		DJJ		JJ	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
0 (basal)	8.3 \pm 1.1	8-12	17.3 \pm 2.9	15-23	8.2 \pm 1.1	6-10	7.9 \pm 1.1	7-11	18.2 \pm 2.8	16-25	8.4 \pm 1.1	6-11
2	8.1 \pm 1.1	7-12	16.9 \pm 2.8	14-22	8.1 \pm 1.1	5-10	8.1 \pm 1.1	8-11	17.9 \pm 2.7	15-24	8.2 \pm 1.1	5-10
6	26.4 \pm 5.9 ^b	20-32	6.9 \pm 1.1 ^b	5-9	8.1 \pm 1.1	5-9	24.2 \pm 5.2 ^b	18-29	33.4 \pm 4.8 ^a	26-38	8.1 \pm 1.1	5-10
10	25.9 \pm 5.8 ^b	21-30	6.8 \pm 1.1 ^b	5-10	8.3 \pm 1.1	6-10	25.4 \pm 5.2 ^b	20-32	32.9 \pm 4.7 ^a	25-36	7.9 \pm 1.1	4-9

^a $P < 0.05$; ^b $P < 0.01$ vs basal levels.

MATERIALS AND METHODS

Subjects

The study comprised of 9 patients (5 women, 4 men, age 33.6 ± 4.2 years, range 28-37). The main complaint was epigastric distension and discomfort after meals of 6-9 years duration (mean 7.3 ± 1.4). They complained also of occasional nausea and vomiting which was spontaneous and sometimes induced epigastric relief. These symptoms occurred 1-1 1/2 h after meals. All of the patients had constipation in the form of infrequent defecation. The patients were medicated by diet regulation as well as gastric antisecretory and prokinetic drugs, but no improvement was achieved.

Physical examination, including neurologic, was normal. The patients had a mean body weight of 63.6 ± 2.1 (range 61-68) kg. Blood count and the results of renal and hepatic function tests and electrocardiography were unremarkable. The patients gave an informed written consent to participate in the study, the details of which and their role in it had been explained to them.

Seven healthy volunteers with a mean age of 34.7 ± 4.3 (range 29-38) years were also included in the study; 4 were women and 3 men. They had no gastro-intestinal manifestations in the past or at the time of enrollment in the study. They gave an informed written consent.

The study was approved by the Review Board and Ethics Committee of the Cairo University Faculty of Medicine.

Methods

The pressure response of the DJJ to DD distension was recorded. The subjects had fasted for 12 h. A condom was applied to the distal end of a Ryle stomach tube (8 French, Pharma Plast Int AS/UK 3540, Lynge, Denmark) containing multiple lateral apertures. A string was tied to each end of the condom so as to fashion a high compliance balloon between them. To the distal end of the tube we applied a silver clip for radiologic control. A mechanical puller for automatic tube withdrawal (9021H, Disa, Copenhagen, Denmark) was used. The empty condom and tube were swallowed by the subjects and the condom was directed to lie in the 1st DD. The tube was connected to a strain gauge pressure transducer (Statham, Oxnard, CA, USA).

Simultaneous measurement of the pressure in the DD, DJJ, and JJ was performed by means of a perfused open-ended tube: 1 mm internal diameter and 1.5 mm

external diameter. One tube was introduced into each of the DD, DJJ, and JJ and connected to a Statham pressure transducer. The position of this manometric tube was accurately determined under fluoroscopic screening for the purpose of which a silver clip had been applied to the distal end of each tube. The pressure recordings were performed 20 min after tubal positioning so that the gut could have adapted to the presence of the tubes.

The resting (basal) pressure was recorded in the DD, DJJ, and JJ. The condom which was lying in 1st DD was filled in 2 mL increments with normal saline up to 10 mL, and the pressures in the DD, DJJ, and JJ were recorded. The condom was then emptied and managed to lie successively in the 2nd, 3rd, and 4th DD. In each of these sites the condom was filled again in increments of 2 mL of normal saline and up to 10 mL, and the pressures in the DD, DJJ, and JJ were registered. We did not exceed the saline fillings of the condom beyond 10 mL for fear of duodenal injury.

Statistical analysis

To ensure reproducibility of the results, the aforementioned recordings were repeated at least twice in the individual subject and the mean value was calculated. The results were adapted statistically using the Student's *t* test, and values were given as mean \pm standard deviation. Differences assumed significance at $P < 0.05$.

RESULTS

Adverse side effects were not encountered during or after performance of the tests, which were completed in all the subjects.

Response of DD, DJJ, JJ pressure to DD distension in the healthy volunteers

The mean resting (basal) pressure in the 1st DD was 8.3 ± 1.1 , DJJ 17.5 ± 2.9 , and in the JJ 8.2 ± 1.1 cm H₂O (Figure 1A, Table 1). The 2nd, 3rd, and 4th DD showed no significant pressure difference against the 1st DD ($P > 0.05$). DD distension with 2 and 4 mL of normal saline did not effect significant pressure changes in the DD, DJJ, and JJ ($P > 0.05$, Figure 1A). Six mL balloon distension of the DD produced significant increase of DD pressure to a mean of 26.4 ± 5.9 cm H₂O ($P < 0.01$), a decrease of DJJ pressures to a mean of 6.9 ± 1.1 cm H₂O ($P < 0.01$) and no significant change in the JJ pressure ($P > 0.05$;

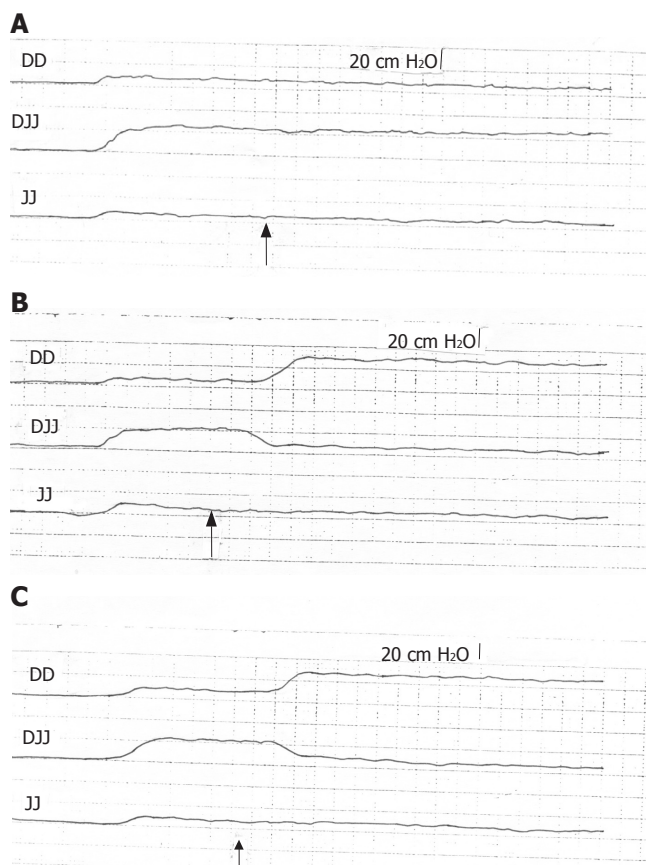


Figure 1 Response of the duodenal (DD), duodeno-jejunal (DJJ), and jejunal (JJ) pressure to 2 mL (A), 6 mL (B) and 10 mL (C) duodenal balloon distension with saline in a healthy volunteer. ↑: duodenal balloon distension.

Figure 1B) and the balloon was dispelled to the JJ. The pressure in the 2nd, 3rd, and 4th DD did not significantly change from the resting pressure. DD balloon distension with 8 and 10 mL of saline produced pressure changes in the DD, DJJ, and JJ similar to those of the 6 mL balloon distension ($P > 0.05$; Figure 1C). When we transferred the collapsed balloon individually to the 2nd, 3rd, and 4th DD and distended the balloon as aforementioned, we obtained results similar to those measured in the 1st DD with no significant difference ($P > 0.05$).

Response of the DD, DJJ, and JJ pressure to DD distension in the patients

The mean resting pressure in the 1st DD, DJJ, and JJ did not differ significantly from that of the healthy volunteers ($P > 0.05$); it recorded 7.9 ± 1.1 for the 1st DD, 18.2 ± 2.8 for the DJJ and 8.4 ± 1.1 cm H₂O for the JJ (Table 1, Figure 2A). The resting pressures in the 2nd, 3rd, and 4th DD were similar to that of the 1st DD ($P > 0.05$). DD distension with 2 and 4 mL of normal saline did not produce significant pressure changes in the DD, DJJ, and JJ (Table 1). Six mL DD balloon distension resulted in a significant increase of the 1st DD pressure to a mean of 24.2 ± 5.2 cm H₂O ($P > 0.01$), an increase of DJJ pressure to a mean of 33.4 ± 4.8 cm H₂O ($P < 0.05$), but no significant JJ pressure changes ($P > 0.05$; Table 1; Figure 2B); the balloon in the 1st DD moved to the 3rd DD. DD balloon distension with 8 and 10 mL of saline produced

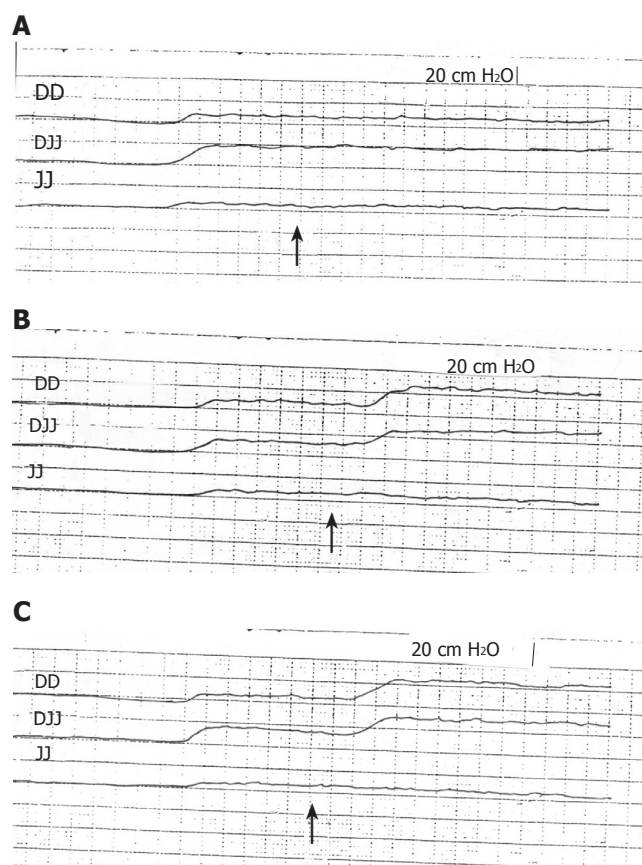


Figure 2 Response of the duodenal (DD), duodeno-jejunal (DJJ), and jejunal (JJ) pressure to 2 mL (A), 6 mL (B) and 10 mL (C) duodenal balloon distension with saline in a patient. ↑: duodenal balloon distension.

pressure changes in the DD, DJJ, and in the JJ similar to those produced by the 6 mL balloon distension ($P > 0.05$, Table 1, Figure 2C). When the balloon in the 1st DD was emptied and transferred to the 2nd, 3rd, and 4th DD, refilled with normal saline as aforementioned, and when the test was repeated in a fashion similar to the one performed in the 1st DD, we obtained similar results with no significant difference ($P > 0.05$).

DISCUSSION

A recent study has demonstrated the presence of a high-pressure zone at the DJJ with a mean length of 1.6 cm^[11]. This zone exists between 2 lower pressure zones: the DD proximally and the JJ distally. It presumably acts as a physiologic sphincter that seems to be responsible for delaying the passage of chyme from the DD to the JJ; this delay has been previously reported by investigators^[7-9].

The stomach contents are delivered to the DD in jets owing to the intermittent opening of the pyloric sphincter^[12]. The chyme boli received from the stomach presumably accumulate in the DD. When they attain a certain volume that distends the DD, a duodeno-jejunal junction inhibitory reflex seems to be evoked which effects DD contraction and DJJ relaxation with a resulting expulsion of the DD contents into the JJ^[11].

The possible existence of a physiologic sphincter at the DJJ apparently regulates the flow of chyme from the

DD to the JJ^[11]. Thus, it seems that the DJJ acts to retain momentarily the DD contents within the DD, supposedly to allow the time necessary for mixing the chyme with the duodenal biliary and pancreatic secretions that are poured into the DD. When this secretions-containing chyme passes to and distends the JJ, the duodeno-jejunal excitatory reflex is apparently evoked with a resulting DJJ contraction^[11]. This reflex seems to act to seal the DJJ upon JJ contraction, probably to avoid reflux of JJ contents back into the DD.

The DJJ pressure response to rising DD or JJ pressure as well as the presence of a high pressure zone at the DJJ would postulate the existence of a physiologic sphincter at the DJJ^[11]. This sphincter seems to dilate on DD contraction, and to contract on JJ contraction^[11]. The response of the DJJ to DD or JJ contraction was postulated to be reflex and mediated through the 'duodeno-jejunal junction reflex'^[11].

The results of the current study have shown that the DJJ did not open upon DD distension with small volumes, thereby presumably retaining the DD contents in the DD for the biliary and pancreatic secretions to act upon. The distended condom was considered to represent a food bolus. In the healthy volunteers the study demonstrated that the 1st DD distension to a certain level affected an increase of the DD pressure, a DJJ opening and the expulsion of the balloon to the JJ. This is in contrast to the patients, in whom DD balloon distension to a degree similar to that of the volunteers produced balloon expulsion to only the 3rd DD; this effect seems to be due to the DJJ failing to open on DD distension. We call the failure of the DJJ to open on DD contraction the 'duodeno-jejunal junction dyssynergia' (DJJDS). This condition had been encountered in 9 of 112 (8.03%) patients who complained of epigastric distension and discomfort after meals.

The failure of the DJJ to open on DD distension might explain the cause of the patients' symptoms. It appears that chyme, after crossing the pyloric sphincter to the DD, gets obstructed at the DJJ. The chyme appears to be retained in the DD and seems to be responsible for the feeling of the epigastric distension and discomfort and vomiting that might follow the meals. We did not specify the pressure level that would initiate the opening of the DJJ. Probably a greater degree of DD distension could have provided the answer, but we refrained from excess balloon distension for fear of duodenal injury.

The question that needs to be discussed now is: what causes the 'duodeno-jejunal junction dyssynergia syndrome' (DJJDS) to develop? Under normal physiologic conditions, the DJJ relaxes on DD contraction, an action mediated through the 'duodeno-jejunal junction inhibitory reflex'^[11]. It appears that DD distension to a certain degree stimulates the mechanoreceptors in the DD wall and the nerve impulses reach the spinal cord. Impulses from the spinal cord supposedly reach the DJJ and effect its relaxation. The DJJDS seems to result from a neurogenic or myogenic defect or a disorder of the neuromuscular transmission. Although the patients were neurologically free, yet further investigations into the pathogenesis of the DJJDS seem to be indicated.

Patients with DJJDS have to be differentiated from patients with functional dyspepsia (FD). The latter patients complain of persistent or recurrent pain or discomfort in the upper abdomen without evidence of organic disease likely to explain the symptoms^[13]. The symptoms have been related to visceral hypersensitivity, impaired gastric accommodation and psychological factors like chronic stress^[14]. However investigations commonly fail to find a cause^[13,14]. Neurohormonal factors might play a role in the pathogenesis of FD. The secretory ability or the metabolic condition of ghrelin may be altered in FD patients, leading to delayed gastric emptying^[15]. Also natriuretic peptides effect inhibitory regulations in gastric motility^[16]. Other disturbances in serum parameters like incretion and adipocytokines may produce abnormal gastric motility, too^[17]. Furthermore, asymmetric geometry of the pyloric orifice in concert with intermittent gastric outflow may affect gastric effluent homogenization with duodenal secretions^[18].

Although the distended condom in the current study was considered to represent a food bolus, yet a study is being planned to investigate the effect of a test meal on pressure recordings in DD, DJJ, and JJ.

In conclusion, the current study has shown that, in contrast to the healthy volunteers in whom the DJJ opens on DD distension, the DJJ did not open in the patients. We call this condition 'duodeno-jejunal junction dyssynergia syndrome' (DJJDS). This condition probably leads to chyme stagnation in the DD and explains the clinical manifestations of the patients. The cause of DJJDS is not known and needs to be studied.

ACKNOWLEDGMENTS

Margot Yehia assisted in preparing the manuscript.

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S- Editor Zhu LH L- Editor Alpini GD E- Editor Yin DH

Histopathological profile of gastritis in adult patients seen at a referral hospital in Kenya

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Received: 2007-03-13 Accepted: 2007-04-04

and intestinal metaplasia. The study shows that inter-relationships between the histological variables in this African population are similar to those found in other populations worldwide including non-African populations.

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Key words: *H pylori*; Gastritis; Stomach; Gastric atrophy; Intestinal metaplasia; Tissue eosinophils; Peptic ulcer; African enigma; Sydney system

Kalebi A, Rana F, Mwanda W, Lule G, Hale M. Histopathological profile of gastritis in adult patients seen at a referral hospital in Kenya. *World J Gastroenterol* 2007; 13(30): 4117-4121

<http://www.wjgnet.com/1007-9327/13/4117.asp>

Abstract

AIM: To conduct a detailed histological study of gastritis in adult patients attending an endoscopy clinic at a Kenyan teaching and referral hospital.

METHODS: Biopsy specimens from consecutive patients were examined and graded according to the Updated Sydney System for *H pylori* infection, chronic inflammation, neutrophil activity, glandular atrophy and intestinal metaplasia. Also documented were gastric tissue eosinophil counts and presence of lymphoid follicles.

RESULTS: The rate of the graded variables, in the antrum and corpus respectively, were as follows: *H pylori* infection (91%, 86%), chronic inflammation (98%, 93%), neutrophil activity (91%, 86%), glandular atrophy (57%, 15%) and intestinal metaplasia (11%, 2%). Lymphoid follicles were noted in 11% of cases. Duodenal and gastric ulcers were documented in 32% and 2% respectively. The mean eosinophil count was 5.9 ± 0.74 eosinophils/HPF and 9.58 ± 0.93 eosinophils/HPF in the corpus and antrum respectively. Significant association was found between the degree of *H pylori* colonisation with chronic inflammation, neutrophil activity and antral glandular atrophy. Biopsies from the antrum and corpus showed significant histopathological discordance for all the graded variables. *H pylori* negative cases were associated with recent antibiotic use.

CONCLUSION: The study reaffirms that *H pylori* is the chief cause of gastritis in this environment. The majority of patients show a moderate to high degree of inflammation but a low degree of glandular atrophy

INTRODUCTION

H pylori has been implicated in the causation of various diseases since the Nobel-winning discovery by Warren and Marshall in 1981^[1]. Ample evidence now exists linking the bacterium to the pathogenesis of chronic gastritis, peptic ulceration, gastric cancers and gastric MALT lymphoma^[2-5]. Over half the world's population is infected with *H pylori* and the infection has been shown to follow a geographic and socio-demographic distribution^[6]. Interestingly however, the infection rate in various populations does not parallel the incidence of morbidity caused by the infection. This has been termed by a number of authors and commentators as the 'African enigma' based on an apparently low incidence of gastric carcinoma and other *H pylori*-associated morbidities in the continent^[7,8]. On the other hand, several commentators and investigators have questioned the realism of this enigma^[9,10]. Both proponents and opponents of the 'Africa enigma', however, concede that there are insufficient data from the continent to allow a more critical analysis of the issue. There is thus a need for more data on gastritis from the African continent.

This study was to provide a detailed histological profile of patients with gastritis at Kenyatta National Hospital (KNH) using the histological criteria of the Sydney system and quantification of gastric tissue eosinophil counts in the endoscopic biopsies. In this study, we present a detailed histopathological assessment of gastritis in an African population within an African setup. There are very few histological studies on gastritis that have been published

from this part of the world. We believe that our findings will further contribute to the available body of world literature on gastritis particularly *H pylori* gastritis.

MATERIALS AND METHODS

Study design

This was a prospective descriptive cross-sectional study conducted on consecutive adult patients as per the inclusion/exclusion criteria. Patients who declined to give consent, those with gastric cancer and those with inadequate biopsies were excluded from the study.

The study was conducted in the Endoscopy Unit of KNH, which is the largest tertiary referral hospital in Kenya. The unit is the only public hospital endoscopy facility situated in the capital city of Nairobi.

Patients

A total of 71 consecutive patients were enrolled in this study between February and May 2004 through the Endoscopy Clinic at KNH. Of the 71 cases enrolled, five were excluded from the study on the grounds of inadequate tissue for proper examination. One patient was found to have normal gastric mucosa on histology and was also excluded from the study as this study was confined to patients with histological evidence of gastritis.

Of the 65 patients with histological gastritis analysed in this study, 32 were males and 33 females (male to female ratio: 1:1). The mean age was 43 years (± 16 years) with a median age of 42 years and an age range of 18 to 86 years. The majority of the patients reported symptoms consistent with dyspepsia (94%). Many of the patients used medication within three months preceding the endoscopy, including antacids in 70%, anti-secretory drugs in 63% (H_2 -antagonists and proton pump inhibitors), antibiotics in 53% and *H pylori*-eradication treatment in 11%. The endoscopists reported gastritis in 77% of the patients, duodenitis in 32%, duodenal ulcer (DU) in 27% and gastric ulcer (GU) in one patient (2%).

Methods

Histological examination was done on formalin-fixed 4 μ m-thick paraffin sections from the corpus and antrum mucosa, 52% of the patients had 3-site biopsies (one from the corpus and two from the antrum including the incisura angularis), while 48% had 2-site biopsies from the antrum and corpus respectively. Five patients did not have histologically-confirmed biopsies from the corpus. The assessment was done on HE-stained sections as per the Sydney System for graded variables of gastritis employing visual analogue scale charts^[11]. Gastric tissue eosinophil count was recorded as the number of cells per $\times 400$ high power field (HPF) using an Olympus Provis AX 70 with a field size of 0.344 mm². Modified-Giemsa stained sections were used for *H pylori* assessment^[12]. The assessment was supervised and checked by an experienced consultant with over 15-year experience. At least 10 cases were randomly reviewed by an independent pathologist as part of quality assurance in the study.

Table 1 Frequency of graded histological variables in patients with gastritis *n* (%)

Variable	Score	0	1	2	3
Chronicity	Corpus (<i>n</i> = 59)	4 (7)	14 (24)	18 (30)	23 (39)
	Antrum (<i>n</i> = 65)	1 (2)	7 (11)	29 (44)	28 (43)
Activity	Corpus (<i>n</i> = 59)	11 (19)	13 (22)	13 (22)	22 (37)
	Antrum (<i>n</i> = 65)	9 (14)	5 (8)	25 (38)	26 (40)
Atrophy	Corpus (<i>n</i> = 59)	50 (85)	7 (12)	2 (3)	0
	Antrum (<i>n</i> = 65)	28 (43)	27 (42)	10 (15)	0
Metaplasia	Corpus (<i>n</i> = 59)	58 (98)	1 (2)	0	0
	Antrum (<i>n</i> = 65)	58 (89)	6 (9)	1 (2)	0
<i>H pylori</i>	Corpus (<i>n</i> = 59)	8 (14)	27 (46)	15 (25)	9 (15)
	Antrum (<i>n</i> = 65)	6 (9)	26 (40)	22 (34)	11 (17)

Statistical analysis

Chi-square test and Mann-Whitney *U* test were used to analyze differences and compare variables between various groups. Wilcoxon signed-rank test was used to compare histological findings between the related variables from the antrum and corpus. The eosinophil counts were transformed to base 10 to make variation constant before subjected to statistical measures of associations. *P* < 0.05 was considered statistically significant (respective values shown). SPSS® version 10 was used for statistical analysis.

RESULTS

Gastric biopsies from 65 patients met the inclusion/exclusion criteria and were analyzed in the study. Most of the biopsies showed a moderate to severe degree of chronic inflammation and neutrophil activity (Table 1).

A total of 59 cases (91%) had histological evidence of *H pylori* infection, 59 (91%, *n* = 65) antrum infection and 56 (86%, *n* = 59) corpus infection. Most of the cases showed a mild degree of colonization by *H pylori* (40% and 46% for the antrum and corpus respectively). Severe colonization was seen in 17% and 15% of the cases respectively. Only 6 cases (9%) were *H pylori* negative, of them 4 recalled a history of antibiotic use within a month preceding the endoscopy but showed moderate to severe inflammation and neutrophil activity in the absence of *H pylori*, 2 having no history of antibiotic use had mild chronic inflammation but no neutrophil activity.

Glandular atrophy in the antrum was seen in 37 cases (57%), of them 27 (42%) were of mild degree and 10 (15%) moderate degree. Severe atrophy was not seen. Glandular atrophy in the corpus was seen in 7 cases (11%). A total of seven cases (11%) had antral intestinal metaplasia, one of them had concurrent mild intestinal metaplasia in the corpus. All but two of the cases showing intestinal metaplasia were of mild degree. No case of severe intestinal metaplasia was noted.

Significant association was found between the degree of *H pylori* colonisation and chronic inflammation in the corpus and antrum (corpus: *P* = 0.012, antrum: *P* = 0.032), as well as between *H pylori* colonisation, neutrophil activity and inflammation (corpus: *P* = 0.000, antrum: *P* = 0.032).

Table 2 Stratifying graded variables in relation to the presence of duodenal ulcer *n* (%)

Histology variable	Site	Category	0	1	2	3	<i>n</i>
Chronic inflammation	Corpus	DU - ve	3 (7)	12 (29)	12 (29)	14 (34)	41 (100)
		DU + ve	1 (6)	2 (11)	6 (33)	9 (50)	18 (100)
	Antrum	Du - ve	1 (2)	6 (13)	21 (45)	19 (40)	47 (100)
		DU + ve	0	1 (6)	8 (44)	9 (50)	18 (100)
Active inflammation	Corpus	Du - ve	10 (24)	12 (29)	5 (12)	14 (34)	41 (100)
		DU + ve	1 (6)	1 (6)	8 (44)	8 (44)	18 (100)
	Antrum	Du - ve	9 (19)	5 (11)	15 (32)	18 (38)	47 (100)
		DU + ve	0	0	10 (56)	8 (44)	18 (100)

DU + ve: patients with duodenal ulcer; DU - ve: patients without duodenal ulcer. ^a*P* < 0.05 *vs* antrum.

Glandular atrophy was significantly associated with *H pylori* colonisation in the antrum but not in the corpus (antrum: *P* = 0.031, corpus: *P* = 0.868). Intestinal metaplasia did not show any significant association with any of the other graded variables.

Biopsies from the antrum and corpus showed significant histopathological discordance as demonstrated using Wilcoxon signed-rank test comparing respective scores. The antrum consistently showed higher grades for all the graded parameters than the corpus: chronic inflammation (*P* = 0.010), activity (*P* = 0.013), intestinal metaplasia (*P* = 0.034) and *H pylori* (*P* = 0.002). Significant discordance was also observed between multiple antral biopsies from patients who had more than one antral biopsy. There was significant discordance for atrophy (*P* = 0.011) in 39 cases showing different scores. A difference in intestinal metaplasia was only recorded in one biopsy not being statistically significant (*P* = 0.317), demonstrating that multiple antral biopsies increase statistical probability for detecting glandular atrophy, but are not helpful for intestinal metaplasia owing to the low rate and degree of intestinal metaplasia.

Duodenal ulcer was significantly associated with higher grades of active inflammation in the corpus (*P* = 0.008) but not in the antrum (*P* = 0.061, Table 2). The association between duodenal ulcer and each of the other graded variables was not significant: chronic inflammation (antrum: *P* = 0.732; corpus: *P* = 0.445), glandular atrophy (antrum: *P* = 0.837; corpus: *P* = 0.527), intestinal metaplasia (antrum: *P* = 0.083; corpus: *P* = 0.504), and *H pylori* (antrum: *P* = 0.498; corpus: *P* = 0.369). No statistically significant association was found between any of the histological and endoscopic parameters with age or gender.

Significant association was demonstrated for chronic inflammation in the antrum with the use of proton pump inhibitors (*P* = 0.048), antibiotics (antrum *P* = 0.028) and eradication therapy (antrum: *P* = 0.023). Patients on these drugs exhibited lower grades of chronic inflammation in the antrum. The association was also significant between chronic inflammation and proton pump inhibitors in the corpus (*P* = 0.027) but not between antibiotics (*P* = 0.240) or eradication therapies (*P* = 0.359). None of the above drugs showed any significant association with the other graded variables including active inflammation. Histamine receptor blockers and antacids did not show any significant association with the presence of duodenal ulcer.

Table 3 Distribution of eosinophils in the antrum and corpus

Eosinophils/HPF	Frequency	
	Antrum	Corpus
0	1	7
1-5	28	31
6-10	15	8
11-15	9	11
16-20	7	3
> 20	6	1
Mean	9.6	5.9
Standard error of mean	0.93	0.74
95% confidence interval	4.42-7.38	7.72-11.74

The mean gastric tissue eosinophil count was 9.6 eosinophils/HPF and 5.9 eosinophils/HPF in the corpus and antrum respectively (Table 3). The highest density observed was 23 eosinophils/HPF in the corpus and 31 eosinophils/HPF in the antrum. Duodenal ulcer was associated with higher eosinophil counts in the corpus (*P* = 0.031). The eosinophil count was significantly associated with chronic inflammation and activity (*P* = 0.000 for both antrum and corpus), as well as *H pylori* colonization (antrum: *P* = 0.002; corpus: *P* = 0.012). Glandular atrophy was not associated with intestinal metaplasia.

Seven (11%) and three (5%) of the patients had lymphoid follicles within the lamina propria of antral and corpus biopsies respectively. Of the three patients with lymphoid follicles in the antrum, two had concurrent lymphoid follicles in the corpus biopsies. Mild dysplasia was found in 1 case and moderate dysplasia in 2 cases respectively, representing 5% of the patients. All these patients had *H pylori* infection.

DISCUSSION

The histopathology results from this study provide further evidence that *H pylori*-associated gastritis is the most common etiopathological type of gastritis among adults presenting to this main referral hospital in Kenya. The rate of 91% is consistent with high prevalence rates reported from previous endoscopic studies in the same hospital. A study around the same period as this study found *H pylori* positivity in 69% of patients with dyspepsia using the rapid urease test^[13]. A previous study in the same setting found a rate of up to 73% and 85% among dyspeptic patients with and without HIV respectively^[14]. Our high pickup rate of *H pylori* gives credence to histology as a sensitive method for diagnosing the bacterium, with the added advantage of enabling morphological assessment of the mucosa. It is, however, noted that antibiotic use prior to endoscopy may affect the sensitivity of histological diagnosis of *H pylori* in gastric biopsies.

The findings in this study reaffirm that *H pylori* is causally associated with chronic inflammation, neutrophil activity and glandular atrophy in the stomach. The findings are similar to those reported from elsewhere around the world. The study also highlights that gastritis in this environment is mainly antral-predominant with significant discordance in the severity of graded variables between

antral and corpus biopsies. The majority of patients undergoing endoscopy in this setting were seen to have moderate to severe gastritis, but much lower rates and severity of glandular atrophy and intestinal metaplasia. These findings are in agreement with the results from several other studies in similar environments^[15]. Studies done in other parts of Africa have shown a mean rate of 28% for glandular atrophy (range 14%-70%) and 14% for intestinal metaplasia (2%-24%) respectively.

The low rate of severe glandular atrophy and intestinal metaplasia is important because progression of these variables has been linked to the development of gastric cancer^[16]. The average age of our patients is much lower than that of patients from areas with a high incidence of *H pylori*-associated gastritis and gastric cancer. Nevertheless, in this study no association was found between the various histological parameters and age. An extensive collaborative study comparing various histological parameters with age and gender matched subjects from other regions would certainly be very informative^[17,18].

Low incidence of gastric cancer is also associated with a low rate of GU as opposed to DU, a low rate and degree of glandular atrophy and intestinal metaplasia, and higher rates of corpus-predominant versus antral predominant gastritis^[19]. In Africa, a wide range of DU: GU ratios have been reported varying from 3:1 to 15-20:1^[9]. In this study, we found a strikingly low rate of GU compared to DU, a low degree of glandular atrophy, a markedly low rate of intestinal metaplasia, as well as antral-predominant gastritis in this population. The DU:GU ratio in this study is 13.5:1. The DU:GU ratio from previous studies in the same setting ranged from 11.5:1 in adults of all ages^[20] to 3:1 in the young and 1:1 in patients over 50 years^[21].

Unfortunately, the data are insufficient to establish the true incidence of gastric cancer in Kenya. However, the indirectly adjusted figures between 1991 and 1993 estimated an annual average crude incidence rate of 7.01 for per 100 000 males and 3.7 for females (compared to a world age-standardised rate, 14.3 for males and 7.1 for females)^[22]. We are thus inclined to believe that the low rate of glandular atrophy, intestinal metaplasia and GU is similar to other parts of the world where the so called Africa enigma has been observed. As yet to be fully determined, host genetic, bacterial virulence and environmental factors underlie this enigma by affecting the progression of infection to neoplasms^[23]. Further study needs to be conducted in order to elucidate the pathological basis of the disparity in the incidence of *H pylori*-associated glandular atrophy, intestinal metaplasia and gastric cancer among various populations of the world.

Lastly, this study demonstrated that gastric tissue eosinophils were significantly associated with the severity of chronic inflammation, neutrophil activity and bacterial colonisation^[24]. Similar observations have also been reported by several other workers including recent experimental studies^[25]. We are of the opinion that the body of knowledge on eosinophils in gastritis has grown sufficiently since the introduction of the Sydney System, and that more attention needs to be given to the utility of quantitative evaluation of eosinophils in gastritis. Because

they are easily identifiable in the gastric mucosa, eosinophil counts may serve as a useful surrogate marker of the severity of inflammation in *H pylori*-associated gastritis.

H pylori is the chief cause of gastritis amongst patients presenting for endoscopy in Kenya. The majority of such patients have a high degree of chronic inflammation, neutrophil activity and *H pylori* infection. Quantitative evaluation of eosinophils may serve as an important surrogate marker of the severity of inflammation in *H pylori* gastritis. Gastritis in this population is significantly antral-predominant, while glandular atrophy, intestinal metaplasia and gastric ulcer are seen to occur at a relatively low rate. Our findings mirror those from other parts of the world with a low incidence of gastric cancer in high *H pylori*-prevalent populations. More studies specifically comparing various histological parameters with age and gender matched subjects from other regions are needed.

ACKNOWLEDGMENTS

The authors thank the entire team of gastroenterologists and endoscopy nurses at KNH for their cooperation, and the KNH Ethics and Research Committee for permission to conduct the study. Part of the study was presented as a poster at the XXVI International Congress of the International Academy of Pathology, September 16-21, 2006, Montreal, Canada.

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S- Editor Zhu LH L- Editor Wang XL E- Editor Yin DH



RAPID COMMUNICATION

Effect of lymph node micrometastases on prognosis of gastric carcinoma

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Received: 2007-04-29

Accepted: 2007-06-04

(range 8-36) mo, Kaplan-Meier survival analysis showed significant improvements in median survival (22.86 ± 3.17 mo, 95% CI: 16.64-29.08 mo *vs* 18.00 ± 7.4 mo, 95% CI: 3.33-32.67 mo) of patients with negative lymph node micrometastases over patients with positive lymph node micrometastases (log-rank, $P < 0.05$).

CONCLUSION: Lymph node micrometastases have a significant impact on the current staging system of gastric carcinoma, and are significant risk factors for prognosis of patients with gastric carcinoma.

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Key words: Gastric carcinoma; Lymph node micrometastases; Prognosis; Stage

Wu ZY, Li JH, Zhan WH, He YL, Wan J. Effect of lymph node micrometastases on prognosis of gastric carcinoma. *World J Gastroenterol* 2007; 13(30): 4122-4125

<http://www.wjgnet.com/1007-9327/13/4122.asp>

Abstract

AIM: To evaluate the relationship between lymph node micrometastases and prognosis of patients with gastric carcinoma and to evaluate the significance of the new assessment of nodal status in determining the pN categories in the 5th edition of the UICC TNM classification.

METHODS: A total of 850 lymph nodes from 30 patients with gastric carcinoma who underwent gastrectomy with lymphadenectomy were assessed by reverse transcription polymerase chain reaction assay in addition to histologic examination. Cytokeratin-20 gene marker was used in this assay.

RESULTS: Routine examination by HE staining confirmed metastasis in 233 lymph nodes from 20 patients. All these 233 lymph nodes were cytokeratin-20 positive. Moreover, lymph node micrometastases were detected in an additional 67 lymph nodes in 12 of these 20 patients. Lymph node micrometastases were also detected in 10 lymph nodes from 2 of 10 patients who had no obvious metastases identified by HE staining. Totally, lymph node micrometastases were identified by the reverse transcription polymerase chain reaction assay in 77 (12.5%) lymph nodes from 14 (46.7%) patients with gastric carcinoma. Of 27 patients who underwent curative resection, 7 (25.9%) were up-staged (from I B stage to II stage in 1 patient, from IB stage to IIIA stage in 1 patient, from II stage to IIIA stage in 1 patient, from IIIA stage to IIIB stage in 1 patient, from IIIA stage to IV stage in 1 patient, from IIIB stage to IV stage in 2 patients). In a median follow-up of 32

INTRODUCTION

Gastric carcinoma is one of the most common malignancies in China and lymph node metastasis is the most important prognostic factor for gastric carcinoma^[1-3]. It was reported that some patients with gastric carcinoma despite undergoing curative resection of the tumor and regional lymph nodes still die of a recurrence^[4-6]. To some degree, the fact may suggest the presence of lymph node micrometastases overlooked by the conventional histopathological examination. Recent advances in immunohistochemical and molecular biologic techniques have made it possible to detect lymph node micrometastases not evidenced by routine HE evaluation^[7-12]. However, the effect of detection of lymph node micrometastases on determining the current staging system and prognosis of gastric carcinoma has not yet been extensively evaluated. Therefore, the aim of this study was to evaluate the significance of the new assessment of nodal status in determining the current staging system of gastric carcinoma. The relationship between lymph node micrometastases and prognosis of patients with gastric carcinoma was also evaluated. In this report, "micrometastasis" in regional lymph nodes was defined as metastasis that was detected only by RT-PCR assay rather than by ordinary HE staining.

MATERIALS AND METHODS

Patients and specimens

The presence of lymph node micrometastases was detected in the 850 dissected lymph nodes from 30 patients with gastric carcinoma who underwent gastrectomy at the Department of Gastrointestinal Pancreatic Surgery, Sun Yat-Sen University of Medical Sciences. There were 17 men and 13 women, ranging in age from 26 to 82 years, with a mean age of 56.8 years. None of these patients received preoperative chemotherapy or radiotherapy. Total gastrectomy was performed in 16 patients, distal subtotal gastrectomy in 13 patients, and proximal subtotal gastrectomy in 1 patient. One patient underwent D1 lymphadenectomy, 22 patients D2 lymphadenectomy, 4 patients D3 lymphadenectomy, and 3 patients palliative resection. According to the Lauren's criteria, 19 tumors were classified as diffusive type carcinomas and 11 tumors as intestinal type carcinomas. Depth of tumor invasion and extent of lymph node metastasis were classified according to UICC TNM classification.

Half of each resected lymph node was fixed in 10% formalin and embedded in paraffin for routine histopathological examination. The other half was stored in 1 mL RNA later (Sigma, USA) at 4°C overnight, then transferred to a clean freezing tube and stored at 70°C for RNA extraction.

RNA extraction

Lymph node samples were homogenized in 1 mL of Trizol reagent (Invitrogen) per 50-100 mg of tissue using a power homogenizer. RNA extraction was carried out according to the protocol recommended by the manufacturer. Total RNA was dissolved in diethylpyrocarbonate-treated water and the volume and quality of RNA were then assessed with an ultraviolet spectrophotometer.

Access RT-PCR

Complementary DNA (cDNA) was synthesized and amplified from total RNA using the Access RT-PCR system (Promega). The primer sequences used for CK-20 detection are 5'-ggctgcgactacatgcatattaca-3' (sense) and 5'-cctcagcagccagtttagcattatc-3' (anti-sense)^[13,14]. cDNA synthesis was monitored by beta-actin RT-PCR using the following primers: 5'-caaatgcttctaggcggact-3' (sense) and 5'-atgctatcacctcccctgtg-3' (anti-sense). RT-PCR was performed in a 25 µL reaction mixture containing 11 µL nuclease-free water, 5 µL 5 × reaction buffer, 0.5 µL dNTP (10 mmol/L), 0.5 µL each of beta-actin primers (20 µmol/L), 1.25 µL each of CK-20 primers (10 µmol/L), 1 µL MgSO₄ (25 mmol/L), 0.5 µL AMV reverse transcriptase (5 u/µL), 0.5 µL Tfi DNA polymerase (5 u/µL), and 3 µL RNA sample. The Access RT-PCR condition was set up as follows: 1 cycle at 48°C for 45 min (reverse transcription), 1 cycle at 94°C for 2 min (AMV RT inactivation), followed by 40 cycles at 94°C for 30 s (denaturation, at 62°C for 1 min (annealing) and at 68°C for 1.5 min (extension), then a final extension at 68°C for 7 min. The resultant cDNA products of CK-20 and beta-actin were 121 base pairs and 381 base pairs, respectively. The RT-PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide.

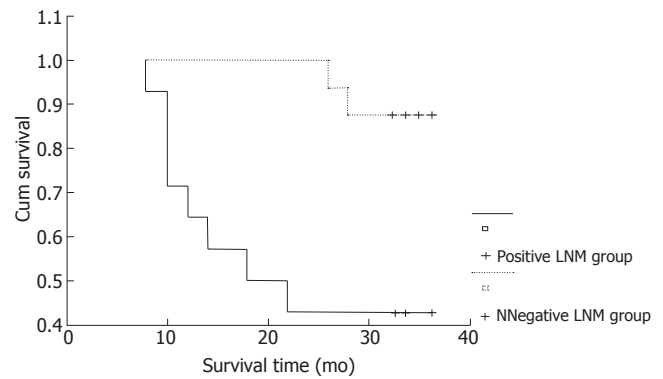


Figure 1 Correlation between lymph node micrometastases and survival of patients with gastric carcinoma (Kaplan-Meier survival analysis).

Statistical analysis

The relationship between lymph node micrometastases and survival of patients with gastric carcinoma was evaluated by Kaplan-Meier survival analysis and log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Routine examination by HE staining confirmed metastasis in 233 lymph nodes from 20 patients. All these 233 lymph nodes were cytokeratin-20 positive. Moreover, lymph node micrometastases were detected in an additional 67 lymph nodes in 12 of these 20 patients. Lymph node micrometastases were also detected in 10 lymph nodes from 2 of 10 patients who had no obvious metastases identified by HE staining. Totally, lymph node micrometastases were identified by reverse transcription polymerase chain reaction assay in 77 (12.5%) lymph nodes from 14 (46.7%) patients with gastric carcinoma. Of 27 patients who underwent curative resection, 7 (25.9%) were up-staged (from I B stage to II stage in 1 patient, from I B stage to IIIA stage in 1 patient, from II stage to IIIA stage in 1 patient, from IIIA stage to IIIB stage in 1 patient, from IIIA stage to IV stage in 1 patient, from IIIB stage to IV stage in 2 patients). In a median follow-up of 32 (range 8-36) mo, Kaplan-Meier survival analysis showed significant improvements in median survival (22.86 ± 3.17 mo, 95% CI: 16.64-29.08 mo *vs* 18.00 ± 7.48 mo, 95% CI: 3.33-32.67 mo) of patients with negative lymph node micrometastases over patients with positive lymph node micrometastases. The difference between these two groups was statistically significant (log-rank, $P < 0.05$) (Figure 1).

DISCUSSION

Despite improved surgical treatment, the prognosis of gastric cancer remains poor currently^[15-19]. pN category is one of the most important prognostic factors for gastric carcinoma. The pN classification of gastric carcinoma, based on the number of metastatic lymph nodes, has been adopted by the current American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC) TNM staging system. According to the AJCC/UICC pN categories, Hundahl *et al*^[20] reported that stage-stratified

5 and 10-year survival rates of patients with gastric carcinoma are as follows: stage I A, 78%/65%; stage I B, 58%/42%; stage II, 34%/26%; stage IIIA, 20%/14%; stage IIIB, 8%/3%; and stage IV, 7%/5%. Kodera *et al*^[21] also reported that the number of metastatic nodes after D2 lymphadenectomy reflects prognosis well and is a strong independent prognostic factor for gastric carcinoma as shown by multivariate analysis. Histopathological examination of resected lymph nodes using HE staining has been the gold standard for diagnosis of lymph node metastasis. However, micrometastases consisting of one to a few cells in lymph nodes resected during gastrectomy are often overlooked by the conventional histopathological method. Therefore, the purpose of this study was to evaluate the significance of detection of lymph node micrometastases in determining the current staging system of gastric carcinoma. The relationship between lymph node micrometastases and prognosis of patients with gastric carcinoma was also evaluated.

Recent advances in immunohistochemical and molecular biologic techniques have made it possible to detect lymph node micrometastases not evidenced by routine HE staining. It was reported that micrometastases are identified in regional lymph nodes from 28%-68.1% of patients^[22-26]. In the current study, we applied the reverse transcription polymerase chain reaction assay to detect micrometastases in the lymph nodes resected from 30 cases of stage I-IV gastric carcinomas. Totally, lymph node micrometastases were identified in 77 (12.5%) lymph nodes from 14 (46.7%) patients with gastric carcinoma. The tumor stage was upgraded in 25.9% (7/27) of patients who underwent curative resection. Similar to our results, Okada *et al*^[13] assessed 435 lymph nodes from 28 patients with gastric carcinoma who underwent gastrectomy with lymphadenectomy using the multiple-marker RT-PCR assay in addition to histologic examination. Of 28 patients who underwent curative resection, the disease stage was upgraded in 10 patients by genetic diagnosis (from Stage I A to Stage I B in 5 patients, from Stage I B to Stage IIIA in 2 patients, from Stage I B to Stage IV in 1 patient, from Stage I B to Stage II in 1 patient, and from Stage II to Stage IIIB in 1 patient). Lee *et al*^[8] applied AE1/3 immunohistochemical staining to detect micrometastases in 3625 regional lymph nodes that were dissected in gastrectomy specimens from 153 patients with early-stage gastric carcinoma (46 patients) and advanced gastric carcinoma (107 patients). Micrometastases were identified in 191 lymph nodes from 75 patients. Twenty-eight of those patients were up-staged. These results indicate that much careful assessment of the lymph node status must be followed in the histopathological examination of resected specimens. Lymph node micrometastases may improve the current staging system of gastric carcinoma and should be validated in future trials as an alternative clinical index.

The prognostic value of lymph node micrometastases for patients with gastric carcinoma is still controversial. Ishida *et al*^[23] reported that gastric carcinomas with micrometastases have significantly worse prognoses at stage II. Lee *et al*^[8] also reported that patients with lymph node micrometastases have a decreased 5-year survival rate (49%) compared with patients without lymph node

micrometastases (76%) for both early and advanced gastric carcinoma. However, Fukagawa *et al*^[27] reported that the presence of immunohistochemically-detected micrometastases in regional lymph nodes does not affect the survival of Japanese patients with pT2N0M0 gastric carcinoma who have undergone gastrectomy with D2 lymph node dissection. The 5-year and 10-year survival rates of patients with and without micrometastases were 94%/79% and 89%/74%, respectively. The differences were not statistically significant ($P > 0.05$). Our results showed significant improvements in median survival (22.86 ± 3.17 mo, 95% CI: 16.64-29.08 mo *vs* 18.00 ± 7.48 mo, 95% CI: 3.33-32.67 mo) of patients with negative lymph node micrometastases over patients with positive lymph node micrometastases. The difference between these two groups was statistically significant (log-rank, $P < 0.05$). These conflicting observations may be explained by the small sample size and selection bias. To draw a further conclusion, larger sample and multi-center investigations on gastric carcinoma are needed.

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S- Editor Zhu LH **L- Editor** Wang XL **E- Editor** Wang HF

RAPID COMMUNICATION

Expression of G3BP and RhoC in esophageal squamous carcinoma and their effect on prognosis

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Supported by Key Science Research Project of Guangdong Province, No. 2003B30104

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Received: 2007-02-03

Accepted: 2007-04-11

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Key words: Esophageal neoplasm; G3BP; RhoC; Immuno-histochemistry; Lymph node metastasis; Prognosis

Zhang HZ, Liu JG, Wei YP, Wu C, Cao YK, Wang M. Expression of G3BP and RhoC in esophageal squamous carcinoma and their effect on prognosis. *World J Gastroenterol* 2007; 13(30): 4126-4130

<http://www.wjgnet.com/1007-9327/13/4126.asp>

Abstract

AIM: To investigate the expression and significance of G3BP and RhoC proteins in esophageal squamous carcinoma (ESC).

METHODS: The expression of G3BP and RhoC proteins in 80 cases of ESC was detected by immunohistochemistry. The relationship was studied between the expression of the two proteins and tumor size, differentiation degree, TNM stage, lymph node metastasis and prognosis of ESC.

RESULTS: The positive expression rate of G3BP in ESC was 71.25%; and the rate in the lymph node metastasis group was significantly higher than that in the non-lymph node metastasis group ($Z = -2.283$, $P = 0.022$), but no relations were found between G3BP expression and tumor size, differentiation degree and TNM stage ($P > 0.05$). The group with G3BP positive expression had shorter survival time than the group with G3BP negative expression ($P = 0.000$). The positive expression rate of RhoC in ESC was 66.25%; and the rate in the lymph node metastasis group was significantly higher than that in the non-lymph node metastasis group ($Z = -2.115$, $P < 0.05$), but no relations were found between RhoC expression and tumor size, differentiation degree and TNM stage ($P > 0.05$). The RhoC positive expression group had a shorter survival time than the RhoC negative expression group ($P < 0.001$). The expression of G3BP protein correlated positively with the expression of RhoC in ESC tissues ($r_s = 0.656$, $P < 0.001$).

CONCLUSION: The expression of G3BP and RhoC protein is closely related to the lymph node metastasis and survival in ESC patients. G3BP and RhoC proteins can be considered as predictors of prognosis in ESC patients.

INTRODUCTION

Many patients with esophageal carcinoma are in intermediate or advanced stage when they see doctors. Because of this, early diagnosis, early treatment and energetic prevention are very important for esophageal carcinoma. The current routine examination methods are insufficient for the diagnosis of small cancer focus and metastatic focus when they are in subclinical stage. But the detection of tumor markers can retrieve the insufficiency of examination methods. The tumor markers not only can be used for the early diagnosis of cancer, but also for post-treatment follow-up, recurrence detection, therapeutic effect evaluation and prognosis monitoring.

G3BP (Ras-GTPase-activating protein SH 3 domain binding protein) and RhoC (Ras homology C) were overexpressed in many kinds of malignant tumors, which was closely related to the invasion and metastasis of tumor cells. Up to date, there has been no study in the relationship between G3BP and esophageal carcinoma. For RhoC, researches have been available in esophageal carcinoma tissues, but without follow-up data^[1]. In the current study, we investigated the expression of G3BP and RhoC proteins in esophageal squamous carcinoma (ESC) by immunohistochemistry, and explored the relationship between the expression of the two proteins and biological behaviour of ESC and post-operative survival time.

MATERIALS AND METHODS

Patients

Eighty ESC patients (65 men and 15 women; mean age 59 years, range 38-84 years) who underwent operation in the Department of Cardiothoracic Surgery of Second Affiliated Hospital to Sun Yat-Sen University were studied. None of the patients had received any preoperative

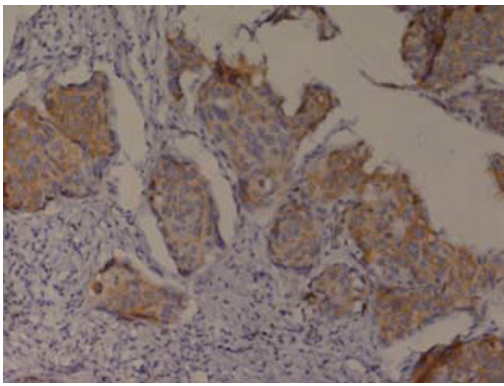


Figure 1 Expression of G3BP protein detected by immunohistochemical staining in esophageal squamous carcinoma (DAB-hematoxylin staining, $\times 200$).

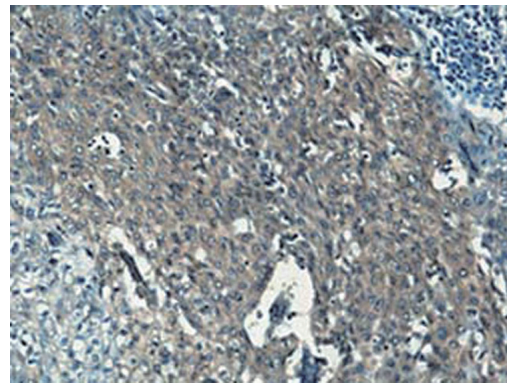


Figure 2 Expression of RhoC protein detected by immunohistochemical staining in esophageal squamous carcinoma (DAB-hematoxylin staining, $\times 200$).

treatment. The follow-up times are more than 28 mo. Of the 80 patients, 58 were dead, and 22 still alive. The postoperative survival time of the dead patients were between 1.5 and 63 (mean 17.2) mo. Tissue specimens were fixed promptly with 100 g/L formaldehyde solution, embedded in paraffin and cut into 4 μm sections.

Immunohistochemistry

The two-step method was used to detect the expression of G3BP and RhoC. The mouse monoclonal antibody against human G3BP was purchased from Department of Pathology, Health Science Center, Peking University, Beijing, China. PowerVision™ Two-Step Histostaining Reagent (PV-6002) and DAB Reagent were purchased from Beijing Zhongshan Golden Bridge Biotechnology Company, Beijing, China. The goat polyclonal antibody against human RhoC was purchased from Santa Cruz Company. The HRP conjugated rabbit anti-goat IgG was purchased from Boster Biotechnology Company.

All sections were deparaffinized and dehydrated with graded alcohol. Endogenous peroxidase was then blocked with 3 mL/L hydrogen peroxide methanol for 10 min at room temperature. Antigen retrieval was performed by treating the slides in citrate buffer in a microwave for 15 min. The sections were cooled for 20 min at room temperature, and then washed with distilled water for 3 \times 5 min. The slides were incubated in a moist chamber with G3BP mouse monoclonal antibody (1:50) and RhoC goat polyclonal antibody (1:100) at 37°C for 2 h. After a complete wash in PBS, the slides were treated with the HRP conjugated goat anti-mouse IgG and the HRP conjugated rabbit anti-goat IgG (1:100) respectively for 25 min at 37°C. After a complete wash in PBS, the slides were developed in 0.05% freshly prepared diaminobenzidine solution (DAB) for 10 min, and then counterstained with hematoxylin, dehydrated, air-dried, and mounted. PBS was used to substitute the primary antibody as a negative control. Positive staining with G3BP and RhoC was defined as brown staining of cytoplasm. The determination was performed by two persons.

Statistical analysis

We used the *t* test for the comparison of tumor size.

The comparison of rate was performed by the rank sum test (Kruskal Wallis and Wilcoxon's test). Postoperative prognosis was evaluated by the Kaplan-Meier method and compared between groups by the log-rank test. The relationship of the expression of G3BP and RhoC was analysed by Chi-square test. Differences were considered as significant when the *P* value was less than 0.05. All statistical analyses were performed using SPSS Win program package 11.5.

RESULTS

Expressions of G3BP and RhoC in esophageal squamous carcinoma

Immunohistochemical staining showed that G3BP was expressed in 57 (71.25%) of 80 cases and RhoC was expressed in 53 (66.25%) of 80 cases (Figure 1 and Figure 2).

Correlation between expressions of G3BP and RhoC protein and clinicopathological features in ESC

The expression rates of G3BP and RhoC protein in the lymph node metastasis group were significantly higher than those in non-lymph node metastasis group ($Z = -2.283$, $P < 0.05$; $Z = 2.115$, $P < 0.05$, respectively), but no relation was found between the two proteins expression and tumor size, differentiation degree and TNM stage ($P > 0.05$) (Table 1).

Correlation between G3BP and RhoC expressions and survival of ESC patients

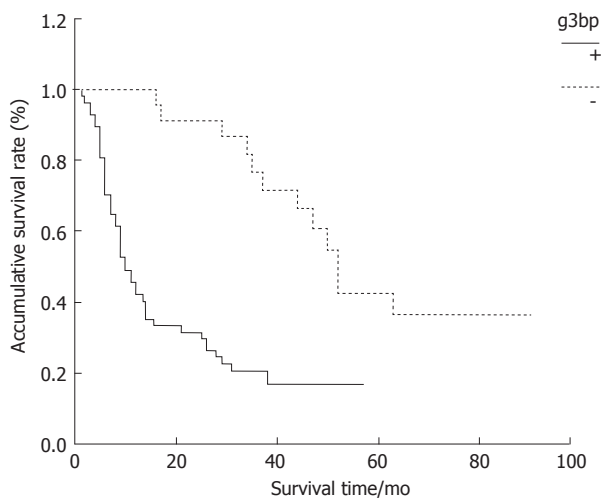
As shown by the Kaplan-Meier curve, both G3BP and RhoC, the overall survival rates of the positive expression group were significantly lower than those of the negative expression group ($P < 0.001$) (Figure 3 and Figure 4).

Correlation between expression of G3BP and RhoC proteins in ESC

Among the 80 cases, 49 cases had positive expression of both G3BP and RhoC, 19 cases had negative expression of G3BP and RhoC, 8 cases had positive expression of G3BP, and 4 cases had positive expression of RhoC. There was positive correlation between the two proteins expression ($r = 0.656$, $P < 0.001$).

Table 1 Relationship between expression of G3BP and RhoC protein and clinicopathological features

Variables	n	G3BP		P	RhoC		P
		-	+		-	+	
Tumor size (cm)		5.0 ± 2.1	5.7 ± 2.9	0.265	5.1 ± 2.0	5.7 ± 3.0	0.396
Differentiation							
Well	43	14	29	0.717	17	26	0.469
Mediate	24	6	18		7	17	
Low	13	3	10		3	10	
TNM stage							
Stage 1	2	2	0	0.083	2	0	0.182
Stage 2	47	15	32		17	30	
Stage 3	23	5	18		6	17	
Stage 4	8	1	7		2	6	
Lymph node metastasis							
No	43	17	26	0.022	19	24	0.034
Yes	37	6	31		8	29	

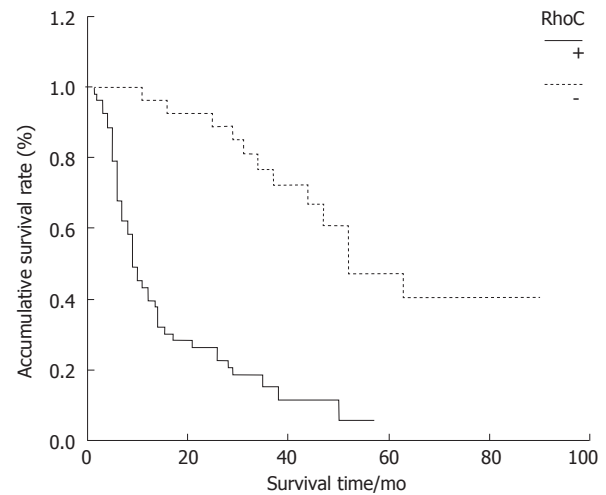
**Figure 3** Relationship between expression of G3BP protein and patients' survival time after operation.

DISCUSSION

Effect of G3BP expression in ESC on clinical prognosis

G3BP was first isolated by Parker from the Chinese hamster lung fibroblasts in 1996^[2]. It is comprised of 466 amino acids, and located in the cytoplasm. Experiment confirmed that G3BP promotes S phase entry in cultured fibroblasts^[3]. According to recent researches, G3BP is the unique protein that could bind with Ras-GTPase-activating protein SH3 domain. The effects of Ras signal transduction pathway in the tumorigenesis and metastasis have been confirmed^[4]. In the Ras signal transduction pathway, Ras-GTPase-activating protein (GAP) attenuated the function of Ras by promoting the hydrolysis from GTP that conjugated with activated Ras to GDP, and then acted as a moderator of Ras signal transduction pathway. The SH3 domain of Ras-GAP was indispensable, for it played an important role in the Ras signal transduction pathway^[5]. Up to now, the proteins that could bind with Ras-GAP have been discovered, such as p62, p190, but they have no relations with SH3 domain^[6,7].

G3BP expresses in many kinds of malignant tumors

**Figure 4** Relationship between expression of RhoC protein and patients' survival time after operation.

in different genetic locums and stages, so it probably participates in the process of tumorigenesis and metastasis. French *et al*^[8] discovered that G3BP expression was specific in human breast cancer tissues and was overexpressed in 88% of tumors examined. Ning *et al*^[9] demonstrated that G3BP was overexpressed in formalin-fixed and paraffin-embedded tissues of some human tumors, such as lung cancer, colon cancer, gastric cancer and breast cancer through immunohistochemical staining. In breast cancer specimens, the degree of G3BP expression correlated positively with the presence of lymph node metastasis. Our study demonstrated that the expression of G3BP had a close relationship with lymph node metastasis, and negative expression group had longer survival time than positive expression group. G3BP may play an important role in the invasion and lymph node metastasis of ESC. The expression of G3BP can be considered as an independant predictor of prognosis of ESC patients. The subtle mechanisms of G3BP inducing tumorigenesis and metastasis remains unknown. Further researches are needed to develop new anti-tumor drugs.

Effect of RhoC in ESC expression on clinical prognosis

RhoC is a member of the Ras-superfamily, and it is an important molecule of the signal transduction pathway. Madaule *et al*^[10] identified a new family of ras genes, the rho genes. It participates in many steps of tumorigenesis and metastasis, such as regulating the cytoskeleton and cell cycle, modulating cell adhesion and movement, impacting cell transformation, facilitating tumor cell genesis, invasion and metastasis, *etc*. RhoC enhances cellular migrating ability by regulating actin-based cytoskeleton, and plays a prominent role in the invasion and metastasis of tumor. Clark *et al*^[11] discovered that the metastatic capacity of melanoma cells was enhanced when the RhoC gene was overexpressed. He proposed for the first time the view that the RhoC gene might be the switch for tumor invasion and metastasis. RhoC expressed in many kinds of malignant tumors. Kleer *et al*^[12] demonstrated that RhoC protein level in tumor tissue was strongly associated with

biologically aggressive invasive carcinomas of the breast. Yao *et al.*^[13] indicated that RhoC GTPase was required for PC-3 prostate cancer cell invasion *in vitro*. Wang *et al.*^[14] demonstrated a positive association between RhoC gene overexpression and tumor invasion and lymphatic metastasis in gastric carcinoma.

Our study demonstrated that the expression of RhoC had a close relationship with lymph node metastasis, and the negative expression group had longer survival time than the positive expression group. RhoC may play an important role in the invasion and lymph node metastasis of ESC. The expression of RhoC can be considered as an predictor of predictoof prognosis of ESC patients. Faried *et al.*^[1] found that RhoA and RhoC proteins promoted both cell proliferation and cell invasion of human oesophageal squamous cell carcinoma cell lines *in vitro* and *in vivo*, and in the two proteins, RhoC was more prominent in enhancing cellular migration. This is coincident with our research. It is significant to further study the role of RhoC in the invasion and metastasis of malignant tumors. RhoC may be considered as an independant predictor of the invasive and metastatic capacity of malignant tumors, and provide powerful assistance for judging prognosis of malignant tumor patients.

Correlation between expression of G3BP and RhoC proteins in ESC

Like other members of the Ras-superfamily, RhoC protein cycles between the active, GTP-bound form and the inactive GDP-bound form. Ras is regulated by GTPase activating proteins (GAPs), which attenuate signaling. The SH3 domain of GAPs is indispensable, for it plays an important role in the Ras signal transduction pathway. G3BP is a unique protein that could bind with Ras-GTPase-activating protein SH3 domain. There was positive correlation between the two proteins expression in ESC. So it is reasonable to presume that G3BP and RhoC proteins promote the invasion and metastasis of tumors through the same pathway and different effective sites.

COMMENTS

Background

Many patients with esophageal carcinoma are in intermediate or advanced stage when they see the doctors. Because of this, early diagnosis, early treatment and energetic prevention are very important for esophageal carcinoma. The current routine examination methods are insufficient for the diagnosis of small cancer focus and metastatic focus when they are in subclinical stage. However, the detection of tumor markers can retrieve the insufficiency of the examination methods. The tumor markers not only can be used for the early diagnosis of cancer, but also for post-treatment follow-up, recurrence detection, therapeutic effect evaluation and prognosis monitoring.

Research frontiers

The effects of Ras signal transduction pathway in the tumorigenesis and metastasis have been confirmed. SH3 domain of Ras-GAP was indispensable, for it plays an important role in the Ras signal transduction pathway. G3BP is a unique protein that could bind with Ras-GTPase-activating protein SH3 domain. G3BP expresses in many kinds of malignant tumors at different genetic locums and different stages. Up to now, no study is available in the relationship between G3BP and esophageal carcinoma. RhoC is a member of the Ras-superfamily, and it is an important molecule of signal transduction pathway. It participates in many steps of tumorigenesis and metastasis, such as regulating cytoskeleton and cell cycle,

modulating cell adhesion and movement, impacting cell transformation, facilitating tumor cell genesis, invasion and metastasis, etc.

Innovations and breakthroughs

The authors investigated the expression of G3BP in esophageal squamous carcinoma for the first time, and confirmed that it is overexpressed in esophageal squamous carcinoma. They discovered that the expressions of G3BP and RhoC proteins have a close relationship with lymph node metastasis and survival in ESC patients.

Applications

G3BP and RhoC proteins can be considered as predictors of prognosis of ESC patients. Further researches about the subtle mechanisms of G3BP and RhoC can provide extensive perspective for the development of new antitumor drugs.

Terminology

G3BP: Ras-GTPase-activating protein SH3 domain binding protein. It is a unique protein that could bind with Ras-GTPase-activating protein SH3 domain specifically. It expresses in many kinds of malignant tumors. RhoC: Ras homology C. It is a member of the Ras-superfamily, and it is an important molecule of signal transduction pathway. It participates in many steps of tumorigenesis and metastasis, such as regulating cytoskeleton and cell cycle, modulating cell adhesion and movement, impacting cell transformation, facilitating tumor cell genesis, invasion and metastasis, etc.

Peer review

This is an interesting manuscript reporting two new immunohistochemical markers (G3BP and RhoC) which appear to serve as prognosticators for esophageal squamous carcinoma. This new information is certainly worthy of publication.

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S- Editor Zhu LH **L- Editor** Ma JY **E- Editor** Lu W

Molecular mechanisms of paclitaxel and NM-3 on human gastric cancer in a severe combined immune deficiency mice orthotopic implantation model

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Supported by Natural Science Foundation of Shanghai, No. 02ZB14072

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Received: 2007-04-26 Accepted: 2007-05-12

Molecular mechanisms of paclitaxel combined with NM-3 on the orthotopic implantation model with human gastric cancer in severe combined immune deficiency mice. *World J Gastroenterol* 2007; 13(30): 4131-4135

<http://www.wjgnet.com/1007-9327/13/4131.asp>

Abstract

AIM: To explore the molecular mechanisms of action of paclitaxel and NM-3 on human gastric cancer in severe combined immune deficiency (SCID) mice.

METHODS: Human gastric cancer cells SGC-7901 were implanted into SCID mice and mice were treated with paclitaxel and NM-3. The effects of paclitaxel and NM-3 on apoptosis of human gastric cancer cells were analyzed using flow cytometry, TUNEL assays, and DNA fragment analyses.

RESULTS: Apoptosis of SGC-7901 cells was successfully induced by paclitaxel, NM-3, and the combination of paclitaxel and NM-3 24 h after injection as shown by the presence of apoptotic hypodiploid peaks on the flow cytometer before G1-S and a characteristic apoptotic band pattern in the DNA electrophoresis. The apoptotic rate detected by TUNEL assay was found to be significantly higher in the paclitaxel/NM-3 compared to the control group ($38.5\% \pm 5.14\%$ vs $13.2\% \pm 1.75\%$, $P < 0.01$).

CONCLUSION: Paclitaxel in combination with NM-3 is able to induce apoptosis of the human gastric cancer cells in SCID mice effectively and synergistically.

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Key words: Gastric cancer; NM-3; Paclitaxel

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INTRODUCTION

Gastric cancer is the common leading cause of cancer death worldwide. Its clinical behavior depends on its capacity to establish metastases of the tumor, and prognosis of advanced gastric cancers is poor. To date, several molecules have been reported to play an important role in gastroenterological tumorigenesis and metastasis^[1-3], but the molecular mechanisms remain to be elucidated^[1-3].

In previous studies using LMD, P²⁷-based RNA amplification, and cDNA microarray, we identified some differentially expressed genes between primary carcinoma cells and lymph node metastatic cells in two patients. Moreover, we further identified four differentially expressed genes in progression of gastric cancer in another group of 15 patients by means of semiquantitative reverse transcribed polymerase chain reaction (RT-PCR), and the expression patterns of these four genes were similar to tumor suppressor genes or oncogenes.

It is now widely accepted that many malignant tumors contain subpopulations of heterogeneous cells. This heterogeneity is exhibited in a wide range of genetic, biochemical and immunologic characteristics. It is likely that specific tumor cells or colonies within the larger heterogeneous tumor specimen are the forerunners of distant metastases^[4]. Thus, many biologic differences might exist between tumor cells in primary and metastatic lesions. Furthermore, the interaction of tumor cells with their living environment may add more differences between these two groups of cells^[2]. As a result, tumor metastasis related genes can be identified by comparing the gene expression profiles between them.

Apoptosis plays a crucial role in the proliferation and turnover of cells in various malignant tumors, and it is enhanced by many anticancer drugs as cytotoxic drugs, hormones, or some recombinant gene, medicine, etc. At present, there is no effective therapy for advanced gastric cancer, as the other malignant tumor, gastric cancer is not only a disease with abnormal cell proliferation and

differentiation, but also a disease with abnormal apoptosis. Thus, the enhanced induction of apoptosis in human gastric cancer cells will be needed to explore. Paclitaxel enhanced the expression of smad3 and smad4 in SCID mice, smad3 and smad4 are kind of multifunction cyclin dependent kinase inhibitors. They play a negative role in cell cycle regulation by inhibiting transition from G0/G1 to S phase. Based on previous studies, we examined the apoptotic indices of human gastric cancer grafted into SCID mice. We investigated its apoptotic effects on human gastric cells, by which we explored its correlated anticancer mechanisms and its synergistic effect combined with NM-3 in order to look for a novel therapy for advanced gastric cancer.

MATERIALS AND METHODS

Materials

RPMI1640 media and TRI201 total RNA isolation kit were purchased from Gibco BRL. The liposome, the trypsin, DMEM culture medium, Hepes and Csc1, 200 bp DNA ladder, dNTP, Taq enzyme and the restriction endonuclease were obtained from Sigma Co.

Drugs and reagents

Paclitaxel was obtained from the Chinese Academy of Science. NM-3 was provided by Doctor Robert, Huston University, USA.

Animal model

Male severe combined immune deficiency (SCID) mice were obtained from Shanghai Experimental Animal Center of Chinese Academy of Sciences. Animals used were 6-7 wk old and weighed 18-22 g. Human gastric cancer SGC-7901 (Shanghai Tumor Institution No: 01842), a poorly differentiated adenocarcinoma cell line, was originally derived from a primary tumor and maintained by passage in the subcutis of nude mice. Animal models were made using orthotopic implantation of histological intact tissue of human gastric carcinoma. Tumors were resected aseptically. Necrotic tissues were cut and the remaining healthy tumor tissues were scissor minced into pieces (about 5 mm × 7 mm in diameter) in Hank's balanced salt solution. Each tumor piece was weighed and adjusted to 50mg. Mice were anesthetized with 4.3% trichloraldehyde hydrate. An incision was made through the left upper abdominal pararectal line. Then the peritoneal cavity was carefully exposed and a part of the serosal membrane in the middle of the greater curvature of the stomach was mechanically injured using scissors. A tumor piece of 150 mg was fixed on each injured site of the serosal surface. The stomach was returned to the peritoneal cavity, and the abdominal wall and skin were closed.

After 12 d, when the tumor reached the size of 0.8-1.0 cm³, mice were randomly separated into four groups, with ten mice per group. Body weight and tumor volume of these orthotopic grafted mice in every group had no obvious difference ($P < 0.05$). Via intraperitoneal injection animals received paclitaxel (5 mg/kg), NM-3 (10 mg/kg), paclitaxel (5 mg/kg) combined with NM-3 (10 mg/kg), or

normal saline, respectively. 24 h later mice were sacrificed by cervical dislocation. Samples of the tumor were immediately frozen in liquid nitrogen for later use. Part of the fresh samples were immediately placed into Eppendorf tubes for flow cytometry analysis.

Ten mg of fresh tissue from every animal was minced with blades to millimeter sizes in tissue medium (RPMI 1640). The supernatant was separated and filtered through a 50-mm nylon mesh. The filtered cells were collected by centrifugation and washed twice with PBS.

Detection of cell apoptosis by TUNEL method

Six cell suspensions (1×10^4 cells) of each group (NM-3, paclitaxel, paclitaxel/NM-3, and saline) were placed separately into 60-mm dishes containing cover glass slides (washed and high-pressure sterilized). Then the glass slides were taken out, washed twice with PBS, and fixed in methanol: freezing acetic acid (3:1) for 30 min. The following procedures were carried out according to the kit instruction. The average number of apoptotic cells was determined by counting 1000 cells on each glass slide and the apoptotic index (AI), i.e. the number of apoptotic cells per 100 cancer cells, was calculated.

Chemical staining of X-gal

1×10^6 /L cells of four groups were fixed by 0.5% glutaral pentanediol for 15 min and washed thrice with PBS. X-gal staining solution (20:1) was added and cells were incubated at 37°C for 4-24 h in a humidified atmosphere containing 50 mL/L CO₂. Blue-stained cells, i.e. those with *LacZ* gene expression, were counted under the microscope and the percentage of the positive cells was calculated.

Detection of the expression of the p27mt gene

5 mg fresh tissue specimens of every mouse in four groups were digested by 0.5 g/L trypsin. The cells were collected and washed twice with PBS. After cell lysis in 500 μL SDS-PAGE cell lysis solution and boiling for 5 min, supernatants were collected after centrifugation. Samples were subjected to western blot analysis.

Detection of cells by flow cytometry

Fresh tissue specimens of the four groups were minced with blades to millimeter sizes in tissue medium (RPMI 1640). Then supernatant was separated and filtered through a 50-mm nylon mesh. The filtered cells were collected by centrifugation, stained, washed twice with PBS, and cell suspension was adjusted to a density of 10^6 /L. 100 μL of cell suspension was mixed with 200 μL of DNA-PREP™ LPR, followed by detection using Coulter Epics XL flow cytometer for 15 min. Cell cycle progression and cell apoptosis rate were analyzed.

DNA fragment analysis

SGC-7901 cells, after being collected and washed twice with PBS, were lysed in 500 μL cell lysis solution [1%Np40, 20 mm/L EDTA, 50 mmol/L Tris-HCl (pH 7.5)] in the presence of 10 μL of protease K. Samples were heated in a 56°C water bath for 1-2 h before extraction with phenol/chloroform. After the DNA precipitate had

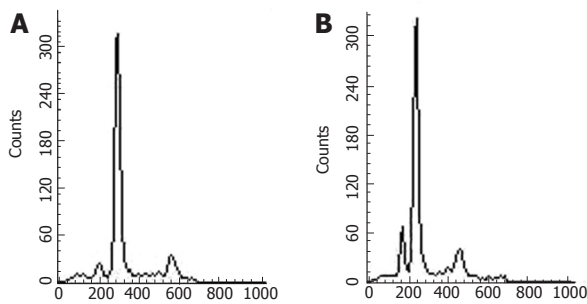


Figure 1 Apoptotic curve line of multidrug-resistant SGC-7901 cells was induced by NM-3 and NM-3 combined with paclitaxel, first apoptotic peak (B) was elevated compared with that of NM-3 group (A).

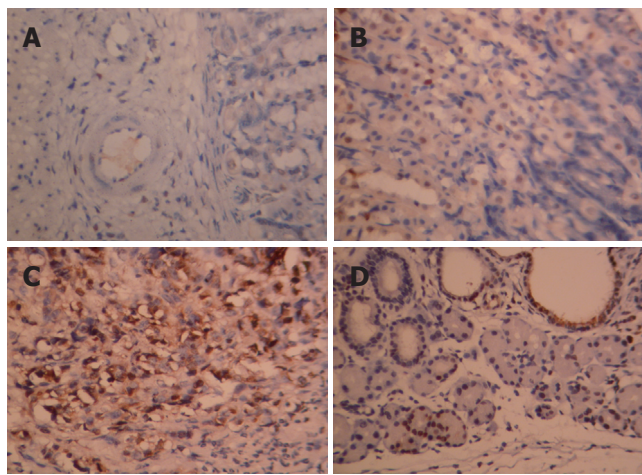


Figure 2 Detection of apoptotic cells by TUNEL method. A: The apoptotic cells appeared yellow in the control group; B: The apoptotic cells appeared yellow in the NM-3 group; C: The apoptotic cells appeared yellow in the paclitaxel group; D: A large number of apoptotic cells appeared in paclitaxel/NM-3 group.

been washed once with 700 mL/L alcohol, 200 μ L of TE was added followed by an overnight incubation with RNase (final concentration 50 μ L/mL) at 37°C. The final DNA was separated by agarose gel electrophoresis (10 g/L) and visualized with the aid of an ultraviolet light lamp.

Statistical analysis

Data were analyzed by *t* test and a *P* value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 11.5 for windows.

RESULTS

Detection of cell apoptosis by TUNEL method

The nucleus of apoptotic cells was dark stained, dark brown and the cytoplasm was concentrated and the cell shrank. The AI of samples of the paclitaxel/NM-3 and the control group was 82.6% (\pm 3.12%) and 5.0% (\pm 0.35%), respectively. This difference was statistically significant (*P* < 0.05) showing that paclitaxel/NM-3 could obviously induce apoptosis of gastric cancer cells (Figure 1 and Figure 2).

Cell cycle progression is shown in Table 1. Whereas

Table 1 Effects of paclitaxel/NM-3 on the cell cycle progression (mean \pm SD)

Group	G ₀ /G ₁	S	G ₂ /M
Control	24.13 \pm 1.12	40.12 \pm 1.09	32.17 \pm 1.48
NM-3	34.42 \pm 3.51	22.41 \pm 2.13	18.23 \pm 3.23
Paclitaxel	26.34 \pm 0.35 ^a	39.20 \pm 0.39 ^a	33.10 \pm 0.89 ^a
Paclitaxel/NM-3	87.42 \pm 3.17 ^b	9.12 \pm 0.22 ^b	15.12 \pm 2.40 ^b

^a*P* > 0.05 vs saline group; ^b*P* < 0.01 vs paclitaxel group).

Table 2 Effects of paclitaxel/NM-3 on the cell apoptosis (mean \pm SD)

Group	AI
Control	13.24 \pm 7.75
NM-3	25.62 \pm 6.46 ^a
Paclitaxel	28.90 \pm 5.38 ^a
Paclitaxel/NM-3	38.51 \pm 5.14 ^b

^a*P* < 0.05; ^b*P* < 0.01 vs control group.

the number of cells in G₀/G₁ phase is lower in the paclitaxel and in the control group compared to the others, the percentage of the S phase cells is higher, indicating that transition time of cell cycle was shortened and cell proliferation was active. However, the percentage of G₀/G₁ phase cells increased and the cell cycle was arrested in G₀/G₁ phase in the paclitaxel/NM-3 group, which was significantly different between the control and the paclitaxel group (Table 1 and Table 2).

X-gal chemical staining:

The adenovirus mediated gene transfer rate was evaluated by X-gal staining. The results showed that the infection efficiency could reach 90%, indicating that recombinant adenovirus could effectively transfer genes *in vitro*.

The expression of p27 protein was evaluated after being injected into the cancer cells with paclitaxel *in vitro*. After the cells that could be used in experiment were injected by paclitaxel/NM-3 for 24 h, these cells were collected and lysed with 1 \times SDS PAGE cell lysis solution. After boiling at 100°C for 5 min, the solution was centrifuged. The supernatant was collected and the protein was detected by TMB system western blot kit, KPL USA. Followed by X-gal chemical staining, there was high expression of a 27 kDa protein in the paclitaxel/NM-3 group while only slight traces were seen (endogenous expression) in the paclitaxel and in the control group. Thus, the human mutant p27 recombinant adenovirus constructed in the present study expresses the p27 gene properly in SGC-7901 cells and the protein product could be expressed at a high level in cells.

PCR identification and effect of paclitaxel/NM-3 on p27mt target gene

The pathological change of SGC-7901 cells and their culture fluid were collected and centrifuged. Five milliliters of the supernatant was mixed with 1 mg of protease K, 2 mL of 1% SDS, 10 mmol/L EDTA and 20 mmol/L

Tris-HCl to be digested for 2 h. After being precipitated by dehydrated alcohol, the DNA was collected. PCR reaction was carried out after adding the forward and reverse primers. Finally, a 275-bp target fragment was amplified, which showed that paclitaxel, paclitaxel/NM-3 had already played an inhibitory role on the p27mt target gene.

Detection of cells by flow cytometry

After the animals had been treated with NM-3, paclitaxel, paclitaxel/NM-3, or saline for 24 h, the detection of apoptotic cells was carried out by flow cytometry and repeated six times. The mean value of hypodiploid cells was: 8.23%, 4.67%, 41.0%, and 1.96%, respectively. Statistical analysis revealed a significant difference between the paclitaxel/NM-3 group and the paclitaxel group and the NM-3 group, (paclitaxel/NM-3 *vs* paclitaxel group, paclitaxel/NM-3 group, NM-3 group and paclitaxel group *vs* normal saline group, $P < 0.01$; paclitaxel group *vs* NM-3 group, $P > 0.05$).

Detection of DNA fragment

The result of DNA electrophoresis showed intact genomic DNA in samples obtained from the paclitaxel and the normal control group while there was an obvious 180-200 bp diploid 'trapezia' pattern in samples derived from NM-3 or the paclitaxel/NM-3 injected group, which was in concordance with the characteristic changes of apoptosis.

DISCUSSION

Since paclitaxel was extracted from Taxul which has special anti-tumor effect, paclitaxel had cell toxic side-effects as Taxol either. We found that paclitaxel could inhibit the activation of CyclinE-CDK2 complex and the activation of CyclinD-CDK4 and CyclinA-C DK2 complex as well. In addition, it could also down-regulate the expression of CyclinB, because the arrest of G₀/G₁ was mainly caused by p27 whose accumulation could be induced by exogenous signal. Gastric tumorigenesis was closely correlated with translocation, deletion and mutation of p27 gene and its expression or activity changes^[2,5]. At present, gastric cancer cells treated by paclitaxel/NM-3 in SCID mice were not reported. If the expression level of p27 mt in gastric cancer cell was down-regulated or inhibited by p27, thus the DNA damaged cell could not transit from G1 phase to S phase directly, that would induced the apoptosis of human gastric cancer cells in SCID mice.

Paclitaxel was first found in 1998, the researchers revealed it effected on cell contact inhibition with TGF- β ^[4]. The degradation of the p27 protein is mainly caused by phosphorylation of the threonine residue at position 187 which is mediated by ubiquitin^[6-8]. Kudo and his colleagues^[9] found that if the 187th threonine of p27 which was mediated by p27 protein, could inhibit cell growth obviously, these inhibitory effects were more obvious on mutant p27 (T187A) than on wild-type p27. The target phosphorylation site of CDK would be protected from phosphorylation. A replication-deficient recombinant adenovirus was constructed which carried p27mt to study apoptosis of the gastric cancer cell line, by which it expected to find a more effective p27 gene

to treat gastric cancer. Koguchi K^[11] reported that they prohibited the viability of the astrocyte when these cells were transfected with exogenous gene p27 carried by adenovirus. Zhang D^[12] reported that the upregulation of the expression of the p27 by retinoic acid significantly inhibited the growth of the oophoroma cell. Koh TY^[13] found high expression of p27 and raised the expression level of cyclinD1 and cyclinE in the cell lines SUN-1066, SUN-1041, SUN-1076 derived from cephalocervical squamous cell carcinoma by transfecting these cells with p27kip1 carried by reconstructed adenovirus, the cancer cells' proliferation were significantly prohibited and the cell cycle analysis showed that the cancer cells were mainly stopped at the G1-S stage in their study. All this showed that p27 was a very important gene related to the development of carcinoma and had significant impact on the onset, development and prognosis of the tumor.

Nowadays, functional reconstruction of anti-oncogene has been a reasonable strategy of gene therapy for tumor. Sasaki T^[14] found that p27mt had stronger suppressive effects than p27wt on apoptosis and cell proliferation when they applied adenovirus-induced p27mt and p27wt to transfect the cholangiocarcinoma cell lines TFK-1 and HuCCT-1. Park KH^[15,16] got the same results when they used adenovirus mediated p27mt and p27wt to transfect lung cancer cell lines NCI H460, NCI H1264, NCI H358 and NCI H157, and a spongioblast line. In our study, mutant p27 was used to transfect gastric cancer cells and the high expression of this gene was proven by p27 polyclonal antibody. This result supported that adenovirus with reconstructed p27mt could transfect the target gene into a gastric cancer cell which derived from human gastric adenocarcinoma tissue, developed into tumor in SCID mice and expressed endogenous p27. By flow cytometry, rate of apoptosis up to 41.0% in paclitaxel/NM-3 group was proven which had significant difference compared to the control group, DNA analysis showed 180-200bp DNA ladder. By TUNEL technique, a value of apoptotic index up to 82.6% was detected which showed significant difference compared to the control group. The results showed that the gene p27 was an important gene related to the occurrence of the large gastric carcinoma and the down-regulation of p27 may be the main cause of cell differentiation dysfunction and apoptosis dysfunction. Upregulating the expression of p27 by mutant p27, which promotes the apoptosis of the tumor cell, could serve as a new scheme in the treatment of advanced gastric carcinoma. Circle analysis showed that the cleavage of tumor cells was stopped at G1 stage via suppressing the activity of the cyclin/CDK kinase by p27mt. Winteringham LH^[17] thought that the accumulation of p27 played an important role in apoptotic mechanisms of the gastric cell cycle arrest at the initiation of cell differentiation. Whether other apoptotic factors might affect this process should be explored further.

Recently, although the apoptosis of the human gastric cancer cell was successfully induced by the application of NM-3 and other gene therapy, but the apoptosis of human gastric cancer cells induced by paclitaxel/NM-3 had not been reported in the SCID mice model^[18,19], it was very useful experimental support of tumor-suppressed function

for human gastric cancer treated by paclitaxel/NM-3. The efficacy of this method and the mechanism of apoptosis strongly indicate that paclitaxel/NM-3 is synergistically effective against human gastric carcinomas.

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S- Editor Liu Y L- Editor Mihm S E- Editor Wang HF

RAPID COMMUNICATION

Effects of nutritional and psychological status in gastrointestinal cancer patients on tolerance of treatment

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Received: 2006-11-16 Accepted: 2006-12-16

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Key words: Gastrointestinal cancer; Chemotherapy; Side effect; Nutritional status; Psychological status; Epidemiological survey

Tian J, Chen ZC, Hang LF. Effects of nutritional and psychological status in gastrointestinal cancer patients on tolerance of cancer treatment. *World J Gastroenterol* 2007; 13(30): 4136-4140

<http://www.wjgnet.com/1007-9327/13/4136.asp>

Abstract

AIM: To assess the effects of poor nutritional and psychological status on tolerance of cancer treatment and the recovery of physical performance status in patients with gastrointestinal cancer.

METHODS: An epidemiological survey with respect to nutritional and psychological status in patients with gastrointestinal cancer was conducted among 182 operated patients in four provincial-level hospitals from December 2005 to June 2006. The food frequency survey method, state-trait anxiety inventory (STAI) and depression status inventory (DSI) were used to obtain information about the diet and psychological status in the patients. Nutritional status in the participants was reflected by serum albumin (Alb), hemoglobin (HB) and body mass index (BMI).

RESULTS: Alb, protein intake and anxiety were associated with the severity of side effects of treatment. The adjusted relative risk (RR) for Alb, protein intake and anxiety was 3.30 (95% CI: 1.08, 10.10, $P = 0.03$), 3.25 (95% CI: 1.06, 9.90, $P = 0.04$) and 1.48 (95% CI: 1.29, 1.70, $P < 0.0001$), respectively. Moreover, calorie intake, HB and depression were associated with the recovery of physical performance status in the patients. Adjusted relative risk was 2.12 (95% CI: 1.09, 4.03, $P = 0.028$), 2.05 (95% CI: 1.08, 3.88, $P = 0.026$) and 1.07 (95% CI: 1.02, 1.12, $P = 0.007$), respectively.

CONCLUSION: Both poor nutrition status and psychological status are independent risk factors for severe side effects of cancer treatment, and have impact on the recovery of physical performance status in patients after treatment.

INTRODUCTION

Chemotherapy and radiotherapy are the two important treatment modalities for cancer and can tumor cells and prolong survival time of cancer patients. Patients receiving high-dose chemotherapy need to be supported with parenteral nutrition^[1]. Nutrition therapy can help cancer patients get the nutrients to maintain body weight and performance status, prevent body tissue from breaking down and rebuild tissues^[2]. Malnutrition can make the patients have more severe chemotherapy-induced toxicity and complications^[3]. High energy/protein diets help patients tolerate the treatment with fewer side-effects^[4,5].

Besides nutrition, psychological status in the patients also impacts treatment outcome. Many cancer patients have psychological problems at different degrees^[6]. Poor psychological status, such as depression or anxiety, not only affects the appetite of patients, but also increases side-effects of treatment^[7] and impacts well-being of patients^[3].

The effects of nutrition on cancer treatment have been studied by many authors^[1-3]. However, few studies have reported on the association between nutritional and psychological status in the gastrointestinal cancer patients prior to chemotherapy/radiotherapy and treatment tolerance^[4,6].

In this paper, we quantitatively analyzed the association between nutritional and psychological status in gastrointestinal cancer patients and treatment tolerance before treatment, and the effects of nutritional and psychological status in patients on physical performance status in the patients after treatment. We also analyzed the relation between nutritional status and psychological status in the patients. Our results may be useful for physicians to

improve the effect of cancer chemotherapy/radiotherapy and the quality of life of patients.

MATERIALS AND METHODS

Materials

Patients with esophagus, stomach, or colorectal cancer admitted to four provincial hospitals in Fuzhou, China, between December 2005 and June 2006 were enrolled in the study. The eligible patients were those who underwent surgical operations and were discharged 20 d before readmission to the hospitals. Patients who had to rely on parenteral nutrition support were excluded. There were 182 eligible participants in the study. After written consent was obtained, in-person interviews were performed for the participants with respect to information on nutrition and psychology status prior to chemotherapy/radiotherapy, side effects of cancer treatment during the treatment, and physical performance status after chemotherapy/radiotherapy. For each participant, the interview was conducted on the next day after admission. The interviewers were trained doctors and nurses in the hospitals. The study was approved by the Institutional Review Board for Human Research in the Fujian Province, China.

Of the 182 participants, 73 were esophagus cancer patients pre-surgery, 62 stomach cancer patients, and 47 colorectal cancer patients.

Methods

The food frequency survey method^[8] was used to obtain information about the diet for each patient over the last week. The daily ten kinds of nutrition intake for each patient in the last week were calculated according to the food composition database^[9]. On the basis of the reference values proposed by the Chinese Nutrition Society^[9], the nutrition intake levels of the patients were evaluated. Daily calorie intake lower than 2400 kcal for male and 2100 kcal for female were defined as the low level of calorie intake, and daily protein intake lower than 70 g for male and 65 g for female were defined as the low level of protein intake.

State-trait anxiety inventory (STAI) and depression status inventory (DSI)^[10] were used to measure psychological status of the participants. Each participant completed these two scales regarding his or her feeling at the time of interview. The patients were considered as suffering from anxiety when their STAI scores were higher than 55, and as suffering from depression when their DSI scores were higher than 40.

Nutritional status of the participants prior to chemotherapy/radiotherapy was reflected by serum albumin (Alb, g/L), hemoglobin (HB, g/L) and body mass index (BMI, BMI = weight/height², kg/m²). Alb in the patient was regarded as deficient when his or her Alb was less than 35 (g/L). HB lower than 120 (g/L) for male and 110 (g/L) for female were defined as abnormal. According to the standard in East China, a person was considered suffering from malnutrition when his or her BMI was lower than 18 (<http://whyuyu.vip.sina.com/news/new000540.htm>).

We examined the side effects of chemotherapy/

radiotherapy which occurred in the gastrointestinal system, respiratory system, liver and kidney, heart, hair, skin and nervous system. The severity of side effects in each system was scored as 1-5, respectively. The scores of side effects were given by the physician within 3-5 d after the start of their first cycle of chemotherapy. Each patient had a total score. The patient had a mild side effect of the treatment when his or her total score was less than 8, and a severe side effect when his or her total score was higher than 9.

When chemotherapy/radiotherapy was over, the nurses scored the physical performance status of the patients in terms of the physical activity status and capabilities of self-care. The score of physical performance status was from 1 to 5. In our analysis, the difference in physical performance status of the patients was determined by the score of physical performance status (score ≤ 2 for poor physical performance status and score > 2 for good physical performance status).

Multivariate logistic regression analysis was used to determine the association between nutritional and psychological status of the patients before and after chemotherapy/radiotherapy. Data were analyzed using SAS version 9.0 software (SAS Institute, Inc., Cary, North Carolina).

RESULTS

There were 55 females with a mean age of 54.3 years (SD = 11.56) and 127 males with a mean age of 55.3 years (SD = 10.96) in the study. The number of patients with stages I-IV of the disease was 15 (8.24%), 27 (14.84%), 69 (37.%) and 71 (39.01%), respectively. Of the 182 patients, 57 (31.32%) had low education levels, 99 (54.39%) middle education levels, and 26 (14.29%) high education levels.

Stepwise logistic regression was used to select the risk factors for the treatment tolerance. At the significance level $\alpha = 0.05$, Alb, protein intake and anxiety were associated with the severity of side effects of the treatment. The relative risk (RR) for Alb, protein intake and anxiety and 95% CI, adjusted for age, gender, stage of disease and tumor location, are shown in Table 1. The adjusted relative risk for Alb < 35 versus Alb ≥ 35 was 3.30 ($P = 0.03$) and 3.25 ($P = 0.04$) for inadequate protein intake versus adequate protein intake and 1.48 ($P < 0.0001$) for anxiety ≥ 55 versus anxiety < 55 . The influence of low Alb on treatment tolerance was higher than that of the high anxiety score.

The association of nutritional and psychological status of the patients with their physical performance status after chemotherapy/radiotherapy was analyzed using stepwise logistic model. At the significance level $\alpha = 0.05$, besides age, gender, stage of disease and tumor location, calorie intake, HB and depression were statistically significant in the model. Adjusted relative risk and 95% CI for these variables are shown in Table 2. Compared with the patients with high level of calorie intake, relative risk for the patients with low level of calorie intake was 2.12 ($P = 0.028$). The hazard of poor physical performance status among the patients with low level of HB was 2.05 times ($P = 0.026$) as high as that among those with normal levels of HB, and the hazard among the patients suffering from

Table 1 Relative risk of severe side effects of chemotherapy and 95% CI for nutrition and psychology factors

Risk factor	Mild side effect		Severe side effect		RR ¹	95% CI
	<i>n</i>	%	<i>n</i>	%		
Alb						
≥ 35	42	63.64	49	42.24	1.00	
< 35	24	36.36	67	57.76	3.30	1.08-10.10
Protein intake						
≥ 70 for male	51	77.27	70	60.34	1.00	
≥ 65 for female						
< 70 for male	15	22.73	46	39.66	3.25	1.06-9.90
< 65 for female						
Anxiety						
< 55	57	86.36	57	49.14	1.00	
≥ 55	9	13.64	59	50.86	1.48	1.29-1.70

¹Relative risk adjusted for age, gender, stage of disease and tumor location.**Table 2** Relative risk of poor physical performance status and 95% CI for nutrition and psychology factors

Risk factor	Poor physical performance status		Good physical performance status		RR ¹	95% CI
	<i>n</i>	%	<i>n</i>	%		
Calorie intake						
≥ 2400 for male	57	50.44	48	69.57	1.00	
≥ 2100 for female						
< 2400 for male	56	49.56	21	30.43	2.12	1.09-4.13
< 2100 for female						
HB						
≥ 120 for male	50	44.25	37	53.62	1.00	
≥ 110 for female						
< 120 for male	63	55.75	32	46.38	2.05	1.08-3.88
< 110 for female						
Depression						
< 40	28	24.78	30	43.48	1.00	
≥ 40	85	75.22	39	56.52	1.07	1.02-1.12

¹Relative risk adjusted for age, gender, stage of disease and tumor location.

depression was 1.07 times ($P = 0.007$) as high as that among those not suffering from depression.

The results suggested that nutritional status and psychological status might affect treatment tolerance and physical performance status of the patients. To examine the independent effects of nutritional status and psychological status, the adjusted relative risk of low BMI and Alb for both anxiety and depression was estimated (Table 3 and Table 4). The patients with anxiety had a higher risk of low BMI and Alb than those with no anxiety (RR = 1.30 and RR = 1.37, $P > 0.05$). Similarly, there was no association between depression and low BMI and Alb. These results suggested that both nutritional status and psychological status might be independent risk factors for severe side effects of the treatment and poor physical performance status of cancer patients.

DISCUSSION

This reports significant association of nutritional and psychological status with the treatment tolerance and the recovery of physical performance status in gastrointestinal cancer patients. Tumors are wasting diseases and many

Table 3 Relative risk of malnutrition for anxiety and 95% CI

	STAI score < 55		STAI score ≥ 55		RR ¹	95% CI
	<i>n</i>	%	<i>n</i>	%		
BMI						
< 18	37	56.06	29	43.94		
≥ 18	77	66.38	39	33.62	1.3	0.64-2.62
Alb						
≥ 35	63	69.23	28	30.77		
< 35	51	56.04	40	43.96	1.37	0.69-2.72

¹Relative risk adjusted for age, gender, stage of disease and tumor location.**Table 4** Relative risk of malnutrition for depression and 95% CI

	DSI score < 40		DSI score ≥ 40		RR ¹	95% CI
	<i>n</i>	%	<i>n</i>	%		
BMI						
< 18	16	23.88	51	76.12		
≥ 18	41	35.65	74	63.35	1.58	0.73-3.40
Alb						
≥ 35	36	39.56	55	60.44		
< 35	22	24.18	69	75.82	1.76	0.86-3.63

¹Relative risk adjusted for age, gender, stage of disease and tumor location.

patients with tumors of the upper gastrointestinal tract have difficulty eating due to side effects of surgery. These patients are weak, tired, and unable to withstand cancer therapies because of malnutrition. The treatment outcome and prognosis of the diseases are associated with the nutritional status of the patients^[11-13]. Chemotherapy-induced toxicity may be more severe in patients with pre-existing malnutrition^[3]. Some studies indicate that high energy/protein diet can reduce side-effects of chemotherapy^[4,5,11,14]. If the patients get enough calories and protein from their diet when they are not on chemotherapy or radiation therapy, they may have a better prognosis and are able to tolerate higher doses of chemotherapy or radiation therapy^[15-17].

Khan *et al*^[4] reported that pre-existing malnutrition in children with acute lymphoblastic leukaemia adversely affect the treatment outcome and decrease the response to chemotherapy. It was reported that decreased tolerance of chemotherapy is associated with poor clinical outcome of malnourished children^[18,19]. However, Sikora *et al*^[20] showed that no significant association was found between nutritional status and side effects of radiotherapy in 45 patients with esophagus cancer. In our study, the prevalence of severe side effects of chemotherapy/radiotherapy among the patients with low level of Alb was 3.30 times as high as that of those with normal Alb, suggesting that malnutrition does not help gastrointestinal cancer patients complete chemotherapy/radiotherapy.

A small amount of protein and calories results in low Alb. In our study, relative risk of severe side effects of the treatment for protein intake was 3.25, showing that protein intake has an independent effect on treatment

tolerance. Because the protein intake was not correlated with calorie intake, no statistical significance was found in the model ($r = 0.86$, $P < 0.0001$). Therefore, the patients who have not gotten enough calories and protein before chemotherapy may have severe side effects of chemotherapy or radiotherapy. Good appetite before cancer treatment may increase patients' energy and improve their sleeping, reducing the side effects of cancer treatment and improving their tolerance to higher doses of chemotherapy or radiotherapy. So, nutrition therapy is a promising, low cost, non-toxic and valid method for improving the outcome of cancer treatment.

Anxiety and depression are normal reactions to cancer. Patients experiencing chronic pain or body function damaged during surgery are at high risk of developing depression or anxiety. Studies showed that the presence of depression or anxiety has a detrimental effect on the recovery from cancer, response to cancer therapy and the death rate of cancer patients^[21,22]. Depression burden significantly influences the severity of side effects of chemotherapy^[23]. Intervention of psychology for the patients can decrease their anxiety symptoms and side effects of the treatment^[24]. Our results also showed that the prevalence of severe side effects of the treatment in patients in good psychological status was lower than that in those with poor psychological status (adjusted RR = 1.48), and there was no association between poor psychological status and low BMI and Alb, suggesting that psychological status is an independent risk factor for cancer treatment tolerance in gastrointestinal cancer patients.

Usually, patients in poor physical performance status have a shorter survival time^[25-27]. Protein and calories are important for providing energy and enhancing physical performance status. Our results showed that pre-existing poor nutrition status of the patients could affect their physical performance status after cancer treatment. Moreover, in our study, 77 (42.3%) patients had low calorie intake and 61 (33.5%) patients had low protein intake. Therefore, in order to promote clinical rehabilitation and improve survival, it is important to provide nutrition therapy and diet advice for the patients.

In the present study, we did not determine nutrition status and psychological status in the patients and study their relation before and after chemotherapy or radiotherapy. Moreover, since other variables reflecting nutritional status of the participants, such as serum pre-albumin, serum transferrin and arm muscle circumference, were not detected, the relative risk of poor nutrition status might be underestimated. Although there are some limitations in our study, our epidemiological survey still provides some important information about the association between side effects of chemotherapy/radiotherapy and nutritional and psychological status of the patients.

In summary, poor nutritional and psychological status are two independent risk factors for severe side effects of cancer treatment, which influence the recovery of physical performance status of the patients after treatment. After diagnosis and operation, the patients should be given diet guidance and mental therapy, which can decrease the severity of side effects of the next treatment and promote

recovery of physical performance status and improve survival.

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S- Editor Liu Y L- Editor Wang XL E- Editor Wang HF

Assessment of T staging and mesorectal fascia status using high-resolution MRI in rectal cancer with rectal distention

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Telephone: +86-21-64041990-2130

Received: 2007-02-27

Accepted: 2007-03-28

Neoplasm; Staging; Mesorectal fascia

Rao SX, Zeng MS, Xu JM, Qin XY, Chen CZ, Li RC, Hou YY. Assessment of T staging and mesorectal fascia status using high-resolution MRI in rectal cancer with rectal distention. *World J Gastroenterol* 2007; 13(30): 4141-4146

<http://www.wjgnet.com/1007-9327/13/4141.asp>

Abstract

AIM: To determine the accuracy of high-resolution magnetic resonance imaging (MRI) using phased-array coil for preoperative assessment of T staging and mesorectal fascia infiltration in rectal cancer with rectal distention.

METHODS: In a prospective study of 67 patients with primary rectal cancer, high-resolution magnetic resonance imaging (in-plane resolution, 0.66×0.56) with phased-array coil were performed for T-staging and measurement of distance between the tumor and the mesorectal fascia. The assessment of MRI was compared with postoperative histopathologic findings. Sensitivity, specificity, accuracy, positive predictive value, and negative predictive value were evaluated.

RESULTS: The overall magnetic resonance accuracy was 85.1% for T staging and 88% for predicting mesorectal fascia involvement. Magnetic resonance sensitivity, specificity, accuracy, positive predictive value, and negative predictive value was 70%, 97.9%, 89.6%, 93.3% and 88.5% for $\leq T2$ tumors, 90.5%, 76%, 85.1%, 86.4% and 82.6% for T3 tumors, 100%, 95.2%, 95.5%, 62.5% and 100% for T4 tumors, and 80%, 90.4%, 88%, 70.6% and 94% for predicting mesorectal fascia involvement, respectively.

CONCLUSION: High-resolution MRI enables accurate preoperative assessment for T staging and mesorectal fascia infiltration in rectal cancer with rectal distention.

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Key words: Magnetic resonance imaging; Rectum;

INTRODUCTION

Rectal cancer is a common disease and a major cause of mortality in Western countries, and the prevalence in China has consistently increased with changes of life style in recent years^[1]. Its poor prognosis is associated with risk both for local recurrence and metastases. The local recurrence is related to the extramural tumor spread into the mesorectum and the tumor distance to the circumferential resection margin (CRM)^[2-4]. With the standardized total mesorectal excision (TME), the overall recurrence rate has been reported to be below 10%, without the help of radiation therapy^[5]. Recent publications have suggested that preoperative radiotherapy enhanced local control^[6-9] and improved prognosis^[6] in T3 and resectable T4 rectal cancer, especially in the patients with involvement of the mesorectal fascia^[8,10]. So accurate preoperative local staging is critical to determine the right patient for preoperative neoadjuvant therapy. High-resolution magnetic resonance imaging (MRI) using the phased coil can visualize the layers of the rectal wall, the mesorectal fascia and predict the depth of tumor invasion, however, the results and techniques were conflicting in the previous reports^[11-13]. Some authors choose not to distend the rectum^[12,14,15] whereas others advocate distension with air or water to improve depiction of the primary tumor^[16-18]. The aim of our study was to assess the accuracy of MRI for preoperative T staging of rectal cancer and the distance to the mesorectal fascia with rectal distention.

MATERIALS AND METHODS

Patients

The study population consisted of 67 patients (37 men and 30 women, with a mean age of 62 years) with histopathologically proved rectal cancer by means of endoluminal biopsy. Rectal cancer was defined as carcinoma within 15 cm of the anal verge. All patients

underwent MRI 1-4 d before surgery. No patient had received preoperative chemoradiotherapy.

MRI examination

MRI was performed using 1.5T whole body system (Magnetom Avanto, Siemens) and a phased array multi-coil in all patients. All patients underwent the hospital's standard cleaning enema procedure. Tepid water was then administered using a rectal enema tube, and the rectum was filled until the patient indicated a sensation of fullness in the rectum. The volume of water ranged from 150 to 400 mL. The rectal tube was removed after completion of instilling the water. No antispasmodic agents were used. The patients were placed in a supine position on an MR table with feet entering MR gantry.

After scout scanning, midline axial and sagittal T₂ weighted turbo spin-echo (T₂W-TSE) images were obtained. The scan protocol was TR 3000-4000 ms, TE 70-90 ms, field of view (FOV) 28-32 cm × 28-32 cm, matrix 276 × 384, slice thickness 5 mm and gap 1 mm. These images were used to plan T₂W-TSE high resolution scans, which were perpendicular to the long axis of the rectum. For a low third rectal tumor, an additional oblique coronal scan along the long axis of the anal canal was also acquired. The scan protocol was TR 2400-3500 ms, TE 90-100 ms, FOV 18 cm × 18 cm, matrix 272 × 320, slice thickness 3 mm and gap 0 mm, in-plane resolution 0.66 × 0.56. The whole examination took about half an hour.

MRI interpretation

Patient's T staging was categorized according to the TNM classification^[4] and was assessed according to the reported criteria (Table 1)^[11]. In our study, T1 and T2 tumors were combined to represent one T stage ≤ T₂, because of limitations at MRI in distinguishing T1 and T2 tumors^[14]. T stage ≤ T₂ tumors were defined as tumors confined to the bowel wall with smooth margins. On the basis of a previous study^[12], tumor with spiculation in the perirectal fat was considered to be a T₃ lesion. The signal intensity of the tumor infiltrating the surrounding structures was thought to be T₄ tumor.

The minimum distance between the outer margin of the tumor and the mesorectal fascia was measured on the Syngo Leonardo Workstation. Measurements for each patient were then categorized into < 2 mm or ≥ 2 mm to assess whether the mesorectal fascia was involved. Two experienced abdominal radiologists who had no clinical and histopathologic information interpreted independently each MR image. Differences in assessment were resolved by means of consensus.

Surgery and histopathologic study

Total mesorectal excision (TME) was performed in 62 patients (anterior resection in 51 patients and abdominoperineal resection in 11 patients, pelvic exenteration in 5 patients). Immediately after surgery, resected specimens were opened on the opposite side of the tumor and fixed in formalin for 24 h. The specimens were then sliced transversely at an interval of 5 mm. The slices were embedded in paraffin, sectioned, and examined histologically after HE staining.

Table 1 Definitions used for staging rectal cancer

Histopathologic examination	MRI
pT1: Tumor invades the submucosa	MRT1: Tumor signal intensity is confined to the submucosal layer
pT2: Tumor invades the muscularis propria	MRT2: Tumor signal intensity extends into the muscle layer, with loss of the interface between the submucosa and circular muscle layer
pT3: Tumor invades through the muscularis propria into the subserosa or into the nonperitonealized pericolic or perirectal tissues	MRT3: Tumor signal intensity extends through the muscle layer into the perirectal fat, with obliteration of the interface between muscle and perirectal fat
pT4: Tumor directly invades other organs or structures or perforates the visceral peritoneum	MRT4: Tumor signal intensity extends into an adjacent structure or viscus

The depth of tumor invasion was classified according to the TNM classification (Table 1)^[4] and the minimum distance between the tumor and the mesorectal fascia was measured macroscopically, and this distance was examined again under microscopy when the margin was assessed macroscopically to be close or involved. If this distance ≤ 2 mm, the mesorectal fascia was considered to be involved^[3,4]. The pathologist was blinded to the result of the MRI findings.

Statistical analysis

Accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for each T stage and predicting CRM infiltration.

RESULTS

Appearance of rectal cancer

The MRI procedure took about half an hour and the patients were well tolerated. The tumors were well visualized in all patients. Forty-two tumors were located in the upper rectum (10-15 cm from the anal verge), 12 in the mid rectum (5-10 cm from the anal verge), and 13 in the distal rectum (less than 5 cm from the anal verge). The size of the resected tumor differed from 0.8 cm × 2 cm to 5 cm × 7 cm (mean 3.5 cm × 4.4 cm). Fifteen mucinous carcinomas were detected with focal (*n* = 8) or diffuse (*n* = 7) high signal (isointense or hyperintense to perirectal fat areas) in the tumor on T₂WI, which was correlated with the mucinous pool on pathologic specimens. In contrast, 52 nonmucinous carcinomas showed isointensity with the normal rectal wall or skeletal muscle on T₂WI.

T staging of rectal cancer

At histopathologic examination, 20 (29.9%) of 67 neoplasms were staged as ≤ pT₂ (Figure 1 and Figure 2), 42 (62.7%) of 67 as pT₃ (Figure 3 and Figure 4), and 5 (7.5%) of 67 as pT₄ (Figure 5). The overall MR accuracy was 85.1%. Over- and under-staging occurred in 9 (13.4%) of 67 patients and 1 (1.5%) of 67 patients, respectively. Accuracy for each T stage was ≤ T₂, 89.6%; T₃, 85.1%; and T₄, 95.5% (Table 2).

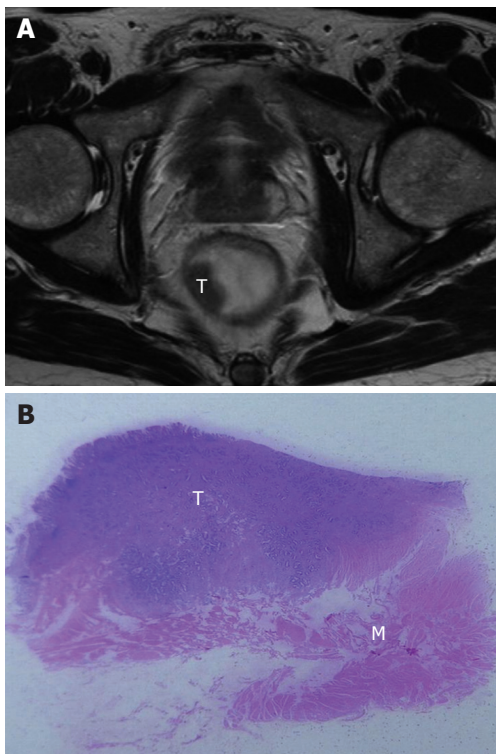


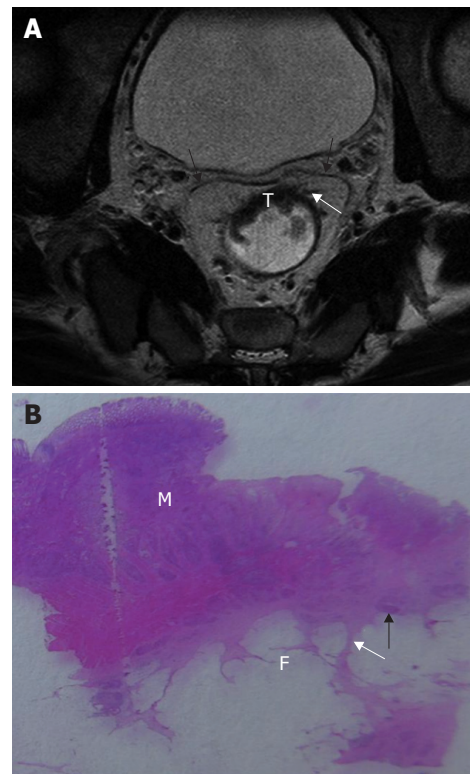
Figure 1 T2-stage rectal cancer in a 58-year-old male patient. **A:** Axial T₂W-TSE MR image (3500/94); **B:** Photograph of the corresponding histopathologic slice (hematoxylin-eosin stain; original magnification, x 10), showed T2-stage tumor (T) that was confined within the muscularis propria (M).



Figure 2 T2-stage rectal cancer overstaged at MR imaging (3000/98) as a T3-stage tumor in a 70-year-old male patient. MR image depicted tumor (T) with spiculations (white arrow) which turned out to be desmoplastic reaction without tumor cells at histology.

Mesorectal fascia status

Mesorectal fascia was visualized on MRI in all patients, which was depicted as a thin, low-signal-intensity structure that envelops the mesorectum and surrounds the perirectal fat. Mesorectal fascia was involved in 15 patients found by pathologists using a cutoff distance of 2 mm between a tumor and the mesorectal fascia^[3,4]. The overall accuracy was 88% for predicting mesorectal fascia involvement (Figures 4B and C). The sensitivity, specificity, positive predictive value, and negative predictive value was 80% (12/15), 90.4% (47/52), 70.6% (12/17) and 94% (47/50), respectively. One false-negative case was due to failure



Figures 3 T3-stage rectal cancer without mesorectal fascia involvement in a 64-year-old female patient. **A:** Axial T₂W-TSE MR image (3000/98) showed tumor in anterior rectal wall (T) with transmurial spiculation (black arrow) from tumor into perirectal fat, and the distance to mesorectal fascia (white arrow) is measured \leq 2 mm; **B:** Photograph of the corresponding histopathologic slice (hematoxylin-eosin stain; original magnification, x 10) confirmed that tumor (black arrows) invaded the perirectal fat (F). Spiculations consist of desmoplastic reactions (white arrow) containing tumor cells (black arrows).

to identify nodal metastases within 2 mm, which is still a diagnostic problem for the radiologist, and the other two cases had more subtle invasion on MRI. Three false-positive cases were anterior rectal tumors (Figure 3A) and the remaining ones had ill-defined margins due to peritumor fibrosis and inflammatory reaction.

DISCUSSION

Colorectal cancer is one of the most common malignant tumors and its incidence is increasing, and rectal cancer is the main part of the colorectal cancer in China^[1]. Rectal cancer has a higher recurrence rate than colon cancer, because of the extensive lymphatic drainage of the pelvis. TME removes the tumor-containing rectum and its draining lymph nodes as a distinct anatomic package, which results in reduced local recurrence rates^[5]. Kapiteijn *et al*^[8] reported that preoperative radiotherapy in combination with standardized TME reduces the local recurrence rate from 8.2% to 2.4% at a 2-year follow-up compared with TME only, but the significant beneficial effect was only observed for T3, T4 or node positive tumors. This study provides strong evidence that patients with T3, T4 or node positive rectal cancer indicated preoperative radiotherapy even when optimal surgery is performed. Recent reports provide convincing evidence of the superiority of preoperative chemoradiotherapy

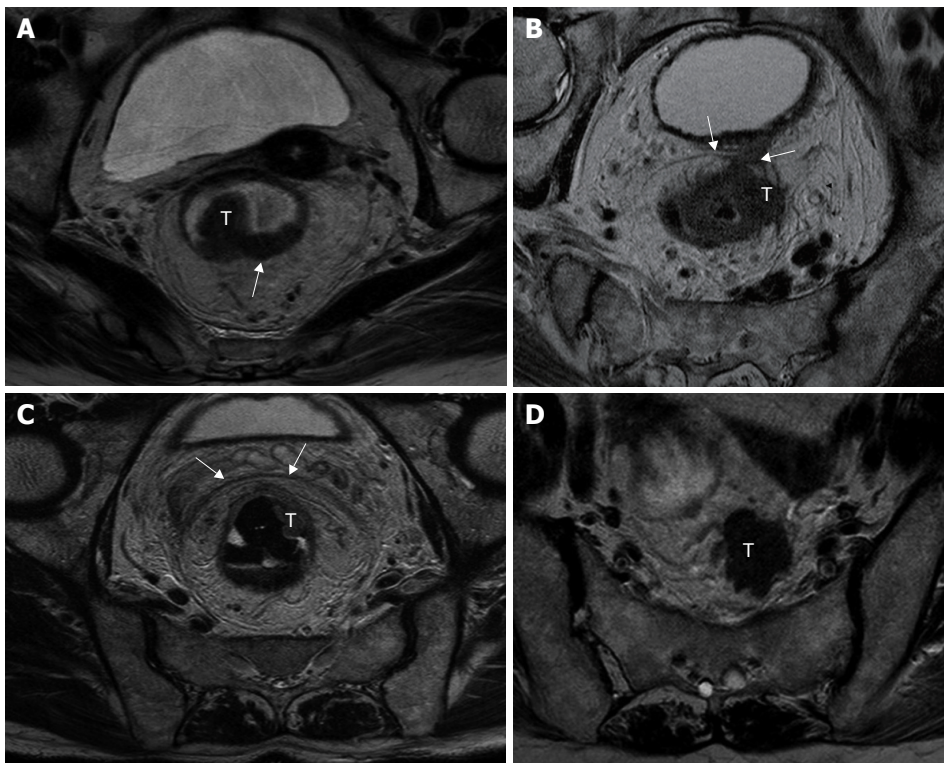


Figure 4 A: T3-stage rectal cancer without mesorectal fascia involvement in a 57-year-old female patient. Axial T₂W-TSE MR image (3000/98) manifested bulky tumor with broad-based nodular (T) with clear margin (white arrows); B: T3-stage rectal cancer with involved mesorectal fascia in a 79-year-old male patient. Axial T₂W-TSE MR image (3000/98) showed tumor (T) extending to mesorectal fascia (white arrows); C: T3-stage rectal cancer with involved mesorectal fascia in a 65-year-old male patient. Axial T₂W-TSE MR image (3000/98) showed heterogeneous strands are noted in the perirectal fat tissues with the thickened mesorectal fascia; D: T3-stage rectal cancer with extramural deposits in a 62-year-old female patient. Axial T₂W-TSE MR image (3000/98) showed extramural deposits (T) in the perirectal space with irregular shape.



Figure 5 T4-stage rectal cancer with fixation to uterus in a 76-year-old female patient. Axial T₂W-TSE MR image (3000/98) showed direct invasion (white arrows) of tumour (T) into uterus (U).

over postoperative chemoradiotherapy^[9,10], especially for patients with a close or involved resection margin at TME^[10]. So precise evaluation of mesorectal fascia involvement is a second important step for patients who are suitable for TME. However, one disadvantage of preoperative chemoradiotherapy is the possibility of over treatment for early-stage tumors. So with the increasing use of neoadjuvant therapy in patients with rectal cancer, accurate staging is needed to avoid unnecessary treatment for early stage tumors.

There have been studies on rectal cancer imaging comparing with endorectal ultrasonography (EUS), computed tomography (CT), and MR imaging^[19,20]. Endoluminal US was considered to be the most accurate modality compared with CT and MR imaging for evaluation of local invasion of rectal cancer, however, it has several limitations: operator dependency; limitation

Table 2 Accuracy for each T stage (n/n)%

	≤ pT2 (n = 20)	pT3 (n = 42)	pT4 (n = 5)
Accuracy	(60/67) 89.6	(57/67) 85.1	(64/67) 95.5
Sensitivity	(14/20) 70.0	(38/42) 90.5	(5/5) 100
Specificity	(46/47) 97.9	(19/25) 76.0	(59/62) 95.2
PPV	(14/15) 93.3	(38/44) 86.4	(5/8) 62.5
NPV	(46/52) 88.5	(19/23) 82.6	(59/59) 100

PPV: positive predictive value; NPV: negative predictive value.

to tumors located in the upper rectum when a rigid probe is used; no assessment of stenotic tumors; and inability to visualize the mesorectal fascia^[19,21]. Although CT was the first technique introduced, it has limitations in differentiating and distinguishing different layers of the rectal wall, and has lower overall accuracy than EUS and MR. But multi-detector row spiral CT scanners, with reconstructions in multiplanar reformations (MPRs), are expected to provide a higher overall accuracy (83%, 34/41). The distance of the tumor to the mesorectal fascia was not assessed^[22]. Studies of conventional MR with body coil in imaging rectal tumors were disappointing because of poor spatial resolution^[23]. High resolution T₂-weighted MRI with phased-array coil is able to depict the detailed anatomy of rectal wall and perirectal structures related to TME^[24], and seems to be the best single method^[21].

Brown *et al*^[11] demonstrated 100% accuracy in the T staging of 28 primary rectal cancers using high-resolution images with a 0.6 mm × 0.6 mm in-plane resolution, however this initial high accuracy and reproducibility were not confirmed. Poon *et al*^[13] reported an overall accuracy of 74% using the similar technique. Our study with a 0.66 mm × 0.56 mm in-plane resolution showed that the

overall accuracy was 85.1% for T staging. Although low-risk T1 tumors may benefit from transanal endoscopic microsurgery (TEM), the clinical value is limited for those who already have a high risk of local recurrence and lymph node metastasis at the time of operation^[25,26]. Differentiation between T1 and T2 tumors may be of little clinical consequence. On the other hand, high-resolution MRI is difficult in distinguishing T1 and T2 tumors, owing to loss of a clear interface between tumor within submucosa and circular muscle^[14]. In addition, as there were few T1 patients ($n = 3$) in our study, we combined T1 and T2 tumors to \leq T2 stage. However, distinguishing T3 from T2 lesions seems to be very important for the use of preoperative therapy and its crucial criterion is infiltration of perirectal fat. The minimal criterion for assignment of pT3 is the absence of any mural muscle between the leading edge of the tumor and the extramural soft tissue. As Brown *et al*^[11] described the presence of tumor signal intensity extending into the perirectal fat signal intensity with a broad-based bulging configuration and in continuity with the intramural portion of the tumor is correlated best with a T3 tumor on MR images^[11], but it is difficult to distinguish spiculation in the perirectal fat caused by fibrosis only from that caused by fibrosis containing tumor cells. Over-staging occurred in 6 (30%) of 20 for T2 tumors in our study, similar to previous report (38.5%, 5/13)^[13], because of extramural fibrosis and inflammatory reaction at the advancing edge of the tumor. It is important to remember that an inflammatory reaction at the expanding tumor margin occurs in about 25% of rectal cancers^[27]. Although Brown *et al*^[11] considered that peritumoral fibrosis has a distinct MR appearance that can be distinguished from the tumor itself, we retrospectively analyzed the over-staging images and failed to show any helpful distinguishing features. So spiculated lesions without preoperative treatment should be designated as T3 rather than T2 disease. In addition, extramural tumor nodules discontinuing from the primary tumor mass that are irregular in shape, are of the T category as pT3^[4]. The irregular shape helps differentiate tumor deposit from lymph nodes with smooth contours.

In T3 tumor, the measurement of the minimum distance between the tumor and the mesorectal fascia is particularly important^[12,14,20]. Histology of resection specimens has shown that the frequency of local recurrence greatly decreases when a tumor-free CRM of more than 1 mm can be obtained^[2]. More recent data have suggested that the risk of local recurrence also is significantly increased with clearances of 2 mm or less. Thus, clearance of 2 mm or less should be considered a positive CRM^[3,4]. Previous reports showed the evaluation of the mesorectal fascia and the CRM on the high-resolution phased-array MRI was highly accurate and reliable, however, the precise measurement is controversial^[12,14,28]. Although these studies were performed without distending rectum, other authors recommended the use of distention to improve tumor visualization and T staging^[16-18]. It is unclear if rectal distention has a detrimental effect on determining the distance between tumor margin and mesorectal fascia. So we distended rectum by administering tepid water of

150-400 mL, and we used the criterion of a cutoff distance of 2 mm between a tumor and the mesorectal fascia for predicting CRM infiltration since rectal distention reduces this distance. If the criteria (a cutoff distance of 6 mm) suggested by Beets-Tan *et al*^[12] were used, many cases would be classified as CRM infiltration. Our performance with rectal distention is comparable with the high performance without rectal distention, with an accuracy of 88%, sensitivity 80% and specificity 90.4%. Torkzad^[29] reported that there is a significant individual variation in the amount of mesorectal fat, which is probably subjected to mechanical pressure from the surrounding structures, so the distance measured from the tumor to the mesorectal fascia could also result in variations. Our study showed that rectal distention did not decrease the distance within 2 mm in most patients, perhaps because tumor invasion and desmoplastic reaction can stiffen adjacent tissues against the effects of the compression, and the amount of mesorectal fat compression is not enough to push the tumor so close to the mesorectal fascia. In our study, the problems in MRI evaluation of mesorectal fascia involvement is (1) the thin perirectal fat in the anterior part leading to difficulties in assessing anterior rectal tumors; (2) ill-defined margins due to peritumor fibrosis and inflammatory reaction leading to unprecise distance measurement; and (3) different volumes of water resulting in various rectal distentions and effects of mesorectal fat compression.

In the differentiation of T3 and T4 tumors, the crucial criterion is infiltration of adjacent structures. The MRI criterion for T4 stage was the obliteration of fat planes between tumor and adjacent organs, but sometimes a loss of fat planes occurred due to an inflammatory reaction or cachexia. Our results showed three T3 lesions were over-staged as MR-T4 disease. In one over-staged patient, the absence of fat planes between the tumor and bladder was due to an inflammatory reaction. The other two patients had large exophytic mucinous adenocarcinoma, pressing the uterus that simulated infiltrating uterus.

There were some limitations in this study. First, no comparison was made between those with and without the rectal distention; Second, the nodal involvement was not assessed, which is still a diagnostic problem for the radiologist depending on morphologic criteria^[20]. Use of ultra-small superparamagnetic iron oxide (USPIO) contrast agents has shown promising results for staging nodal metastases, but need further evaluations^[30]. Third, different volumes of water were used due to varying tolerance of the patients.

Notwithstanding these limitations, high-resolution MRI enables accurate preoperative assessment for T staging and mesorectal fascia infiltration in rectal cancer with rectal distention.

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S- Editor Zhu LH L- Editor Ma JY E- Editor Lu W

Dedifferentiated liposarcoma arising from the sigmoid mesocolon: A case report

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Received: 2007-04-10 Accepted: 2007-04-30

Abstract

Dedifferentiated liposarcoma is a variant of liposarcoma with a more aggressive course. It occurs most commonly in the retroperitoneum and rarely in other anatomic locations. In the present report, we describe a case of dedifferentiated liposarcoma that occurred in an unusual location, sigmoid mesocolon, which has not yet been documented.

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Key words: Dedifferentiated liposarcoma; Sigmoid mesocolon

Winn B, Gao J, Akbari H, Bhattacharya B. Dedifferentiated liposarcoma arising from the sigmoid mesocolon: A case report. *World J Gastroenterol* 2007; 13(30): 4147-4148

<http://www.wjgnet.com/1007-9327/13/4147.asp>

INTRODUCTION

Liposarcoma is a common sarcoma of the soft tissue in adults, occurring most commonly in the extremities and retroperitoneum^[1]. Well-differentiated liposarcoma is the most common variant. Dedifferentiated liposarcoma is a variant of liposarcoma with a worse prognosis, and occurs most commonly in the retroperitoneum^[1,2]. Although well-differentiated liposarcoma arising from the sigmoid mesocolon has been documented^[3-5], to the best of our knowledge, dedifferentiated liposarcoma from this unusual location has not been reported. This report describes such a case in a 59-year-old male.

CASE REPORT

A 59-year-old previously healthy male presented as an outpatient with complaints of fatigue, recent 20 pounds weight loss, loss of appetite, and abdominal fullness. Physical examination revealed an abdominal mass in the left upper quadrant. CT scan of the abdomen showed a 19 cm × 17 cm × 12 cm mass with a necrotic center and an irregular soft tissue periphery. The mass was in close approximation to the splenic flexure of the colon, immediately anterior to the pancreas, and displaced the stomach medially. A left hemicolectomy, and splenectomy along with excision of the tail of the pancreas were performed. Gross examination revealed a 25 cm grey, fleshy mass with a necrotic center involving the wall of the left colon apparently arising from the sigmoid mesocolon (Figure 1A). An ulcer was present in the center of the large bowel segment, likely from pressure effect (Figure 1A). A 13 cm × 10 cm × 7 cm homogenous pale yellow, fatty mass was present at the periphery corresponding to the well differentiated component, composed of numerous lipoblasts and fibrous septa (Figure 1B). At the interface between the fatty and solid areas, the histology showed an abrupt transition from a well-differentiated component to a dedifferentiated component resembling a high-grade malignant fibrous histiocytoma (MFH) (Figure 1C). The dedifferentiated component was composed of pleomorphic, spindle cells with elongated, hyperchromatic nuclei, admixed with tumor giant cells, and abundant necrosis. The mitotic index was two per high power field and atypical mitoses were easily identified. The dedifferentiated component invaded into the wall of the colon (Figure 1D). Immunolabeling for S-100 was positive in the well-differentiated component (as would be expected of a liposarcoma). CD117 (C-KIT) and CD34 were negative in the dedifferentiated component, thus ruling out a gastrointestinal stromal tumor (GIST). Based on the histology and immunoprofile, a diagnosis of dedifferentiated liposarcoma was rendered.

Two and a half months postoperatively, a CT scan showed a recurrent mass for which the patient underwent resection of the posterior gastric wall and distal pancreatectomy. The pathologic examination revealed a mass (9.5 cm × 5.0 cm × 5.0 cm), which was morphologically identical to the patient's previous tumor. Following adjuvant radiation therapy the patient was doing relatively well two years after the diagnosis without evidence of recurrent tumor.

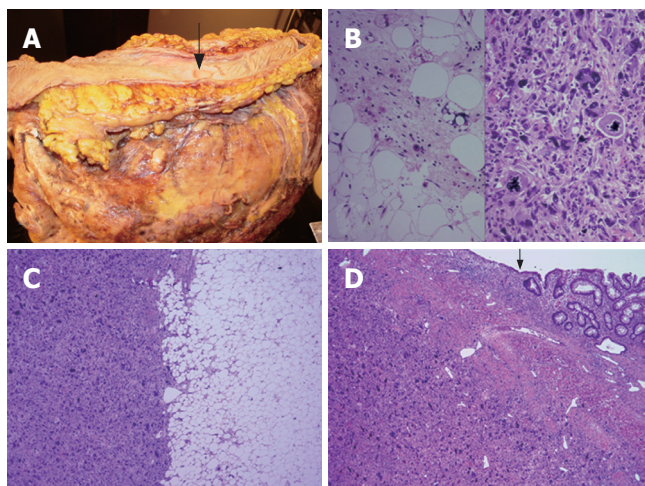


Figure 1 A: Gross picture showing a 25 cm dedifferentiated liposarcoma arising from the sigmoid mesocolon. Notice the bowel mucosa is unremarkable except for a small ulcer (arrow); B: The well-differentiated component showing numerous lipoblasts and fibrous septae. The dedifferentiated component resembles a malignant fibrous histiocytoma with pleomorphic nuclei and tumor giant cells (HE, x 400); C: The area showing an abrupt transition from the well-differentiated component to the dedifferentiated component (HE, x 40); D: The dedifferentiated component invading into the bowel wall. The arrow points to the overlying bowel mucosa (HE, x 40).

DISCUSSION

Liposarcoma is divided into five subtypes according to the World Health Organization (well differentiated, dedifferentiated, myxoid, pleomorphic, and mixed type)^[6]. Both well-differentiated and dedifferentiated liposarcomas have an equal sex predilection with the highest incidence in the 6th-7th decade of life. Dedifferentiated liposarcomas are defined histologically by a transition from well-differentiated liposarcoma to a non-lipogenic sarcoma with variable histological grade^[6]. The dedifferentiated component can resemble any sarcoma, but often mimics MFH like our case^[2]. Interestingly, dedifferentiated liposarcoma, despite its high-grade histology, has a less aggressive clinical course than other types of high-grade sarcoma, although the underlying mechanism is unclear^[6]. Compared to well-differentiated liposarcoma, dedifferentiated liposarcoma has similar genetic changes, ring or giant marker chromosomes, but has a worse prognosis^[6]. Approximately 40% of dedifferentiated liposarcomas will recur locally, 17% will metastasize, and 28% of the patients will ultimately die as a result of the tumor^[6]. Therefore, it is important to thoroughly sample the resected mass in order to identify the non-lipogenic component, which may only comprise a small portion of the tumor. Also sampling of the usually adjacent innocuous looking fatty component is essential as this often contains the well-differentiated liposarcoma component.

Dedifferentiated liposarcoma has been reported most commonly in the retroperitoneum, rarely in other anatomic

locations^[2]. Five cases of dedifferentiated liposarcoma have been described from the small bowel mesentery^[8]. Although well-differentiated liposarcoma has been documented in the sigmoid mesocolon^[3-5], dedifferentiated liposarcoma from this unusual location has not been reported. Another unusual feature about our case was the dedifferentiated component transmurally invading the bowel wall.

The location of the lesion in our case raises the possibility of a GIST. GISTs usually resemble smooth muscle tumor with a variety of histological patterns, which does not consist of a well-differentiated liposarcoma component, necessary for the diagnosis of a dedifferentiated liposarcoma. GISTs typically stain with CD117 and CD34^[9]. Immunohistochemically, dedifferentiated liposarcoma is usually negative for CD117 and CD34 in the dedifferentiated component and positive for S100 protein in the well-differentiated component. Dedifferentiated liposarcoma needs to be distinguished from other high-grade sarcomas such as MFH because these high-grade sarcomas have a much worse prognosis^[7]. On needle biopsies the distinction of dedifferentiated liposarcoma from other high-grade sarcomas can be problematic as the samples are usually small, and the component of dedifferentiated liposarcoma can be identical to other high-grade sarcomas.

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S- Editor Liu Y L- Editor Wang XL E- Editor Wang HF

Clearance of hepatitis C virus after living-donor liver transplantation in spite of residual viremia on end date of interferon therapy before transplantation

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Received: 2007-04-14 Accepted: 2007-05-12

Abstract

Interferon (IFN) therapy is the only treatment strategy for hepatitis C virus (HCV) infection after liver transplantation (LT), but prophylactic and treatable IFN therapy after LT has been shown to be insufficient due to the adverse effects of IFN and ribavirin. In this paper, we describe the disappearance of HCV after LT without IFN therapy in the presence of residual viremia on the day of LT. We herein report our findings since this is considered an important case for the anti-HCV strategy of post LT. A 60-year old woman with LC and HCC was referred to Nagasaki University Hospital in August 2004. After she underwent LT on February 18, 2005, we injected peg-IFN- α -2a the 11th time at 18 wk and HCV-RNA was still positive in the serum at LT. The serum HCV-RNA was negative one month after operation and subsequently dissolved 15 mo after operation without IFN therapy. As a result, we speculate that if HCV-RNA is positive while HCV core antigen is negative before LT, then it may lead to clearance of HCV after LT. Therefore long acting peg-IFN- α -2a is thus considered a potentially effective agent for the treatment of HCV-related cirrhosis before LT.

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Key words: Pegylated interferon; Liver transplantation; Hepatitis C virus

Ichikawa T, Nakao K, Hamasaki K, Honda T, Shibata H, Akahoshi M, Eguchi S, Takatsuki M, Kanematsu T, Eguchi

K. Clearance of hepatitis C virus after living-donor liver transplantation in spite of residual viremia on end date of interferon therapy before transplantation. *World J Gastroenterol* 2007; 13(30): 4149-4151

<http://www.wjgnet.com/1007-9327/13/4149.asp>

INTRODUCTION

Living donor liver transplantation (LDLT) has become a common treatment strategy for hepatocellular carcinoma (HCC) and end stage liver cirrhosis (LC) in Japan^[1]. However, hepatitis C virus (HCV) infection, the most common cause of LDLT, is found in nearly all re-infected graft livers, thus leading to a rapid progression to LC and re-liver transplantation^[2]. Interferon (IFN) treatment for HCV infection after LT is the only treatment strategy at present, but its effects are still incomplete^[3]. Because the titer of HCV is the most decay in early transplant phase of liver transplantation^[4], anti-HCV therapy could thus be considered at this time^[5], but prophylactic and treatable IFN therapy after LT has so far been ineffective due to the adverse effects of IFN and ribavirin^[6].

Recently, pegylated interferon (peg-IFN), utilizing polyethyleneglycol moiety attached to IFN *via* an amide bond, has been used in the treatment of chronic HCV infection. Peg-IFN- α -2a characterized by a prolonged absorption half-life (50 h), a restricted volume of distribution (8-12 L), and a decreased clearance (94 mL/h)^[7], has been found to be safe and tolerable after LT^[8]. We thus consider peg-IFN- α -2a a potentially useful treatment of LC due to HCV infection in patients awaiting liver transplantation.

In this case, we made an attempt to achieve HCV clearance from a graft liver after LDLT. For this purpose, peg-IFN- α -2a mono-therapy was performed for 13 wk until LDLT. We observed the disappearance of HCV after LDLT without IFN therapy in the presence of residual viremia on the day of LDLT. The titer of HCV disappeared in early post-LDLT due to the administration of peg-IFN- α -2a. We herein report our findings.

CASE REPORT

A 60-year old woman with LC and HCC was referred

to Nagasaki University Hospital in August 2004. She was diagnosed having diabetes and HCV-related LC in 1995 and 1999, respectively, and had no history of blood transfusion, alcohol abuse and intra-venous drug use. After the diagnosis of LC, she was treated with IFN- α , but this medication was stopped due to depression. In August 2003, a tumor was detected measuring 3.5 cm in diameter in the caudate lobe of the liver, she underwent trans-arterial chemoembolization (TACE) therapy twice in September 2003 and February 2004. The HCC decreased and no new HCC was detected. However, she suffered from hepatic encephalopathy (disorientation and flapping tremor) in June 2004 and thus was hospitalized. Consequently she and her family decided to undergo living donor liver transplantation at our hospital.

She entered our hospital for evaluation of LDLT in September 2004 (Figure 1). On admission, she had no ascites or hepatic encephalopathy. Laboratory data revealed 2.0 mg/dL total bilirubin, 97 IU/L AST, 88 IU/L ALT, 855 IU/L ALP, 6.7 mg/dL total protein, 3.1 mg/dL albumin, 76% prothrombin time and 81 000/ μ L platelets, she was thus evaluated to be Child-Pugh grade B. Her HCV genotype was 1b, and the viral load in serum was 1860 KIU/mL by Amplicor PCR or 3320 fmol/L by HCV core antigen assay. HCC remained unchanged on admission. She underwent peg-IFN- α -2a treatment with the goal of virus clearance, because LDLT might be unnecessary depending on the status of her liver function. We concluded that LDLT should be performed after disappearance of HCV-RNA in her serum. She received peg-IFN- α -2a, 90 μ g once a week from December 12, but we had to discontinue it due to neutropenia (under 500/ μ L) after the first week of therapy. As a result, we modified the regimen of peg-IFN- α -2a treatment from once a week to once every two weeks.

At five weeks after initiation of peg-IFN- α -2a treatment, HCV core antigen was not detectable in her serum, but HCV-RNA was still detectable by qualitative PCR(nested PCR) even at 12 wk after initiation of therapy. We therefore decided to perform LDLT with her daughter as donor, since the complete disappearance of HCV-RNA was thought to be impossible. She underwent LDLT on February 18, 2005, and we used peg-IFN- α -2a for the 11th time at 18 wk and HCV-RNA was still positive in the serum at this time.

Surgery was successfully performed. Histopathological examination revealed that the explanted liver exhibited mixed macro and micro nodular cirrhosis, and three tumors were observed in the caudate lobe, one of them showed complete necrosis by TACE while the others were viable and diagnosed with well-differentiated HCC, and another well-differentiated HCC was found in the anterior segment of the explanted liver. There was no-evidence of vascular invasion of HCC. The patient was given tacrolimus and predonisolone as immunosuppressants. On postoperative day (POD) 2, she underwent re-anastomosis of the hepatic artery, because of decreased arterial flow caused by an intimal tear. On POD 5, she underwent the third laparotomy, because of hematoma around the portal vein and thrombosis in the portal vein. Due to food aspiration, methicillin resistance staphylococcus aureus (MRSA) caused pneumonia and a systemic infection. At

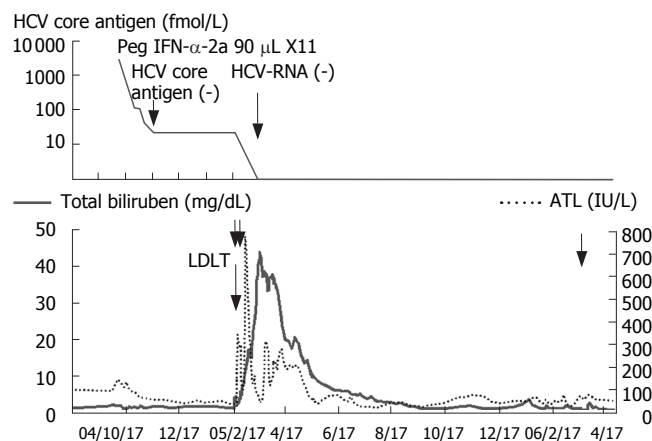


Figure 1 Clinical course of the disease at our hospital. The upper diagram showing the course of the virus titer, in which HCV is the core antigen (fmol/L), and expediently negative HCV core antigen is denoted as 20 fmol/L and negative qualitative HCV-RNA as 1 fmol/L. The lower diagram showing the course of total bilirubin and AST. Three small arrows show abdominal operation after LDLT and a large arrow demonstrates LDLT.

this time, icterus was worsening and the total bilirubin in serum rose to 30 mg/dL on POD 30. She was treated with intensive care, tracheostomy, artificial respiration, MRSA specific antibiotics, hyperbaric oxygen therapy and steroid pulse therapy. Finally, at post operative month (POM) 4, she was weaned off artificial respiration and her total bilirubin decreased to 3.1 mg/dL and she showed a good recovery at POM 8. At POM 13, she was operated on for anastomotic stricture of the common bile duct and was then treated on an out-patient basis.

Serum HCV-RNA was negative at POM 1 and subsequently dissolved at POM 15. Liver biopsies performed at POD 2, 5, 32 and POM 13 revealed no findings of HCV reactivation.

DISCUSSION

Crippin JS and colleagues^[9] tried IFN- α -2b (1 or 3 MU/d three time a week) +/- ribavirin (400 mg) within 12 weeks, and a loss of detectable HCV-RNA was seen in 5/15 (33%) patients. Thomas RM and colleagues^[10] reported that 12 cases (60%) responded to IFN- α -2b 5 MU/d therapy before LT with a clearance of serum HCV-RNA, four of the 12 cases did not show any evidence of HCV recurrence after LD. Fornis X and colleagues^[11] used IFN- α -2b 3 MU/d + ribavirin 800 mg/d and 9 cases (30%) demonstrated a clearance of serum HCV-RNA, 6 of the 9 cases did not have any evidence of HCV recurrence after LD. Recently, Everson GT and colleagues^[12] described that low dose IFN treatment, peg-IFN- α -2b 0.5 μ g/kg per week or IFN- α -2b 1.5 MU/d + ribavirin 600 mg/d per six months for genotypes 2 and 3 or 1 year for genotype 1, was performed for advanced HCV patients, and 12 of 15 cases showing a clearance of HCV-RNA before LT remained HCV-RNA negative 6 or more months after transplantation and 32 cases who were positive for HCV-RNA before remained HCV-RNA positive. In a previous report^[13], IFN therapy before LT was shown

to be an effective treatment for the clearance of HCV with advanced LC, but sustained HCV clearance after LT was never acquired in patients with a detectable level of HCV before LT. In contrast, our case demonstrated a clearance of HCV-RNA regardless of the fact that HCV-RNA in serum was positive before LDLT. After liver transplantation, we did not prescribe cyclosporine A and mycophenolate mofetil exerting anti-HCV effects *in vitro*. We hypothesize that peg-IFN- α -2a as a long acting IFN, when injected immediately prior to LDLT, may thus induce an anti-viral activity in the anhepatic phase and also soon after LDLT. The HCV titer is the lowest in the anhepatic phase and the immediately early post LDLT^[4], therefore this period requires treatment in order to achieve a clearance of HCV. In our case, HCV showed trace quantities due to the negative HCV core antigen and positive HCV-RNA. We thus speculate that trace quantities of HCV in the graft respond to the continuing presence of peg-IFN- α -2a, though a similar case has not been reported up to now.

In cases of a sustained viral response to IFN therapy after LT, hepatic fibrosis does not progress except for other reasons of hepatic injury, rejection and stenosis of bile^[14,15]. HCV relapse post SVR after LT has been reported at 7, 8 and 15 mo after treatment^[15,16]. For the reasons stated above, we must pay attention to advanced liver fibrosis and HCV-RNA in the serum.

Based on our findings, we hypothesize that positive HCV-RNA is positive and negative HCV core antigen before LT may lead to a clearance of HCV after LT, and long acting peg-IFN- α -2a may be a potentially effective agent for HCV infection before LT. This hypothesis should be further confirmed.

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S- Editor Liu Y L- Editor Wang XL E- Editor Liu Y



CASE REPORT

Plasmapheresis and corticosteroid treatment for persistent jaundice after successful drainage of common bile duct stones by endoscopic retrograde cholangiography

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Received: 2007-03-20 Accepted: 2007-04-04

Abstract

Prolonged cholestasis is a very rare complication of endoscopic retrograde cholangiography (ERC). Only few cases with this complication are reported in the English literature. We report persisting cholestatic jaundice in a 73-year old man after successful therapeutic ERC for choledocholithiasis. Serologic tests for viral and autoimmune hepatitis were all negative. A second-look ERC was normal also. He denied any medication except for prophylaxis given intravenous 1 g ceftriaxone prior to the ERC procedure. After an unsuccessful trial with ursodeoxycholic acid and cholestyramine for 2 wk, this case was efficiently treated with corticosteroids and plasmapheresis. His cholestatic enzymes became normal and intense pruritis quickly resolved after this treatment which lasted during his follow-up period. We discussed the possible mechanisms and treatment alternatives of intrahepatic cholestasis associated with the ERC procedure.

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Key words: Endoscopic retrograde cholangiography; Cholestasis; Plasmapheresis

Saritas U, Aydin B, Ustundag Y. Plasmapheresis and corticosteroid treatment for persistent jaundice after successful drainage of common bile duct stones by endoscopic retrograde cholangiography. *World J Gastroenterol* 2007; 13(30): 4152-4153

<http://www.wjgnet.com/1007-9327/13/4152.asp>

INTRODUCTION

The main complications of endoscopic retrograde

cholangiography (ERC) and sphincterotomy are bleeding, pancreatitis, perforation and cholangitis^[1,2]. The wide variety of miscellaneous complications of diagnostic and therapeutic ERC have also been described in the endoscopic literature, usually as single case reports, including splenic trauma, portal venous air, gall-stone ileus, intra-abdominal hemorrhage from injury to the gastropiploic artery, bleeding from bile duct varices and necrotizing fasciitis^[3-8]. Similarly, prolonged cholestasis is a very rare complication of ERC^[9,10] and a concern for a retained common bile duct stone, inadequate biliary drainage or another cause of liver disease. We describe a case of prolonged cholestatic jaundice with intense pruritis following successful therapeutic ERC in the absence of a retained stone, inadequate biliary drainage or unrelated cause of liver disease. This case gave a prompt response to corticosteroid treatment and plasmapheresis.

CASE REPORT

A 73-year-old male was admitted with complaints of epigastric pain and jaundice for the last few days. Physical examination was normal except for the apparent icterus. His laboratory results indicated elevated serum bilirubin levels (total: 18.3 mg/dL, direct fraction: 12.9 mg/dL), high alanine aminotransferase (ALT: 153 U/L, normal up to 40 U/L), alkaline phosphatase (ALP: 349 U/L, normal up to 130 U/L) and gammaglutamyl transpeptidase (GGT: 272 U/L, normal up to 40 U/L). An upper abdominal ultrasonography revealed dilated biliary tree and calculi of various sizes with associated acoustic shadows in the gall bladder. He underwent ERC procedure. Half an hour prior to ERC, a prophylaxis with 1 g intravenous ceftriaxone was done. During the procedure, a cholangiography revealed multiple filling defects consistent with stones and debris in the dilated common bile duct. A standard sphincterotomy was performed with gallstones and debris obstructing the common bile duct removed endoscopically. Control with balloon occlusion cholangiography was normal. However, during the days following ERC, a progressive increase of total and conjugated bilirubins (27.3 mg/dL and 15.5 mg/dL, respectively) associated with intense pruritis was noted. Nevertheless, the patient had no fever and his leukocyte count was within the normal range. ERC was repeated due to concern for a retained stone and cholangiography obtained at that time was normal. To ascertain bile duct drains well enough, we

placed a plastic biliary stent into the common bile duct. However, the insertion of biliary stent did not improve his jaundice. Three days after the second ERC, the patient continued to have intense pruritus and elevated cholestatic biochemical tests. Ten days later, his total and direct bilirubin levels were 28.7 mg/dL and 17.2 mg/dL, respectively and alkaline phosphatase 743 U/L. An upper abdominal ultrasound was unrevealing. Hepatitis B surface antigen and anti-hepatitis C virus antibody, anti-nuclear and anti-mitochondrial antibodies were all negative. There was no improvement in his pruritus and blood tests despite treatment with ursodeoxycholic acid (15 mg/kg per day) and cholestyramine (12 g/d) for 2 wk. He refused to undergo a liver biopsy. Cholestyramine and ursodeoxycholic acid were stopped and we started on treatment with prednisone (40 mg/d). Although his serum bilirubin (10.7 mg/dL) and ALP (181 U/L) levels gradually declined, his annoying pruritus continued. Biliary stent was removed endoscopically and plasmapheresis was performed. After plasmapheresis, pruritus promptly declined. Prednisone was stopped. His liver function tests were normal within 1 mo and remained stable after a further 4 mo period of follow-up.

DISCUSSION

Prolonged cholestasis is a rare complication of ERC^[3,4]. Our patient developed prolonged cholestasis following successful drainage of the bile ducts with ERC procedure. Viral, mechanical, and immunologic etiologies were excluded confidently in the present case. The only possible risk factor regarding the etiology of cholestasis aggravated after ERC was the administration of ceftriaxone before the procedure.

The cause of prolonged cholestasis after a successful ERC is unclear. Four similar patients have been reported previously, three of them recovered completely after treatment with glucocorticoids^[3,4]. Some speculated that it might have been caused by allergy to the radio-contrast material or antibiotics used during ERC leading to impaired secretion of bilirubin^[4]. Others also postulated that the radio-contrast material used may be the responsible factor for cholestatic injury after ERC as these materials are capable of acting toxically on hepatocytes with disruption of the canalicular plasma membrane^[3]. Thus, it may be reasonable to treat such patients with ursodeoxycholic acid and glucocorticoids. If we cannot get a symptomatic control of such complaints in these cases, plasmapheresis can be an optimal alternative treatment modality which has been successfully used to treat intractable pruritus associated with cholestatic diseases^[11,12]. Our patient's complaints abruptly improved soon after plasmapheresis sessions. Although liver biopsy examination would be

valuable to make a differential diagnosis better in the setting of intrahepatic cholestasis, our patient refused to undergo liver biopsy procedure. In the differential diagnosis of refractory cholestasis in this case, stent-induced cholangitis or cholestasis should also be evaluated. However, our case had no classic signs of stent-induced cholangitis, including fever and right upper quadrant pain. Moreover, sequential treatments with ursodeoxycholic acid/cholestyramine and prednisone which were partially successful in our case might not be useful in the setting of cholestasis or cholangitis associated with the presence of biliary stent.

We believe that intrahepatic cholestasis should be considered in the differential diagnosis of persistent jaundice after ERC when a retained common bile duct stone and other causes of liver disease are excluded. Plasmapheresis can be a therapeutic choice in these patients with persistent pruritus and biochemical abnormalities despite treatment with ursodeoxycholic acid, cholestyramine and glucocorticoids.

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S- Editor Zhu LH L- Editor Wang XL E- Editor Liu Y



CASE REPORT

Liposarcoma of the stomach: A rare case report

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Received: 2007-04-06 Accepted: 2007-04-26

Abstract

Liposarcoma is the most common soft tissue sarcoma and accounts for 15%-20% of all mesenchymal malignancies. The tumor occurs most frequently in the limbs, retroperitoneum and rarely has a visceral location. We report a case of a gastric liposarcoma in a male patient. A 68 years old male patient was admitted to hospital for abdominal discomfort and fullness lasting for a month. He reported rare episodes of vomiting. The CT examination revealed a large epigastric mass (8 cm x 4 cm) involving the lesser curvature of the stomach, in contact with the pancreas and gallbladder. Fatty areas within the mass were evident. A total gastrectomy together with cholecystectomy was performed. The histopathological diagnosis was a well differentiated liposarcoma. The patient did not undergo any adjuvant treatment, he is under close follow up and two years later he is disease free. We report this case due to the rarity of this tumor in the stomach (nine cases reported in the literature).

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Key words: Gastric liposarcoma; Lipoblasts; CT diagnosis; Total gastrectomy; Well-differentiated type

Tepetes K, Christodoulidis G, Spyridakis ME, Nakou M, Koukoulis G, Hatzitheofilou K. Liposarcoma of the stomach: A rare case report. *World J Gastroenterol* 2007; 13(30): 4154-4155

<http://www.wjgnet.com/1007-9327/13/4154.asp>

15%-20% of all patients with sarcoma. The hallmark of liposarcoma is the immature fat cells or lipoblasts. It is usually localized in the limbs, retroperitoneum and trunk. Viscera rarely are involved. Histologically, liposarcomas are subdivided into-differentiated, myxoid, round cell and pleomorphic^[1] sarcomas. Gastric liposarcomas are very rare and only a few (nine) cases have been reported worldwide^[2-4]. We report a case of a large epigastric mass proven to be a gastric liposarcoma.

CASE REPORT

A 68-year old male patient was admitted to the surgical ward with symptoms of abdominal pain and discomfort localized in the epigastrium during the last month. He reported rare episodes of vomiting and weight loss of more than 8 kg in that period. He had not any specific past medical history. At physical examination a palpable mass was found in the epigastrium and the right upper quadrant. Blood tests showed a mild leucocytosis and tumor marker values were normal. Plain abdominal X-ray showed no specific findings. Computed tomography (CT) examination revealed a large epigastric mass (9 cm × 4 cm) arising from the gastric fundus and protruding beyond the gastric wall, being in contact with the pancreas and gallbladder. Heterogeneous fatty density areas were demonstrated. No hepatic metastasis or nodal involvements were detected. An upper GI endoscopy revealed a mucosal lesion and the posterior gastric wall seemed to be compressed (Figure 1). The patient underwent a laparotomy. During laparotomy the pancreas was found to be free of tumor involvement and there were fibrous adhesions of the duodenum to the gallbladder. A total gastrectomy was carried out due to the size of the lesion in order to achieve negative margins, cholecystectomy was also performed. Histopathological examination revealed a well-differentiated liposarcoma. The tumor was basically composed of mature adipocytes which varied in size and shape and had enlarged atypical hyper chromatic nuclei. Uni- and multivacuolated lipoblasts were sparsely distributed throughout the lesion. Mitotic activity was generally very low, ranging from 0 to 1 mitosis per 10 high-power fields (Figure 2).

The patient did not undergo any adjuvant treatment, he was under close follow-up including biannual CT scanning and upper GI endoscopy at the time we wrote our report and 2 years later he was disease free.

INTRODUCTION

Liposarcoma is a common soft tissue tumor occurring in

DISCUSSION

Liposarcoma is primarily a tumor in adults with a peak

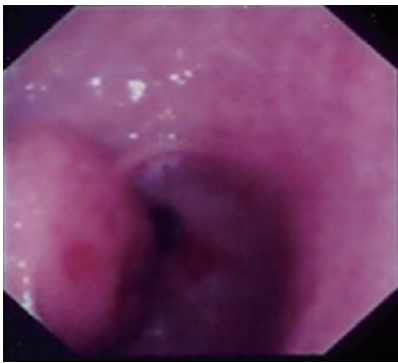


Figure 1 Upper GI endoscopy showing a mucosal lesion in the gastric antrum being suspected for lipomatous tumor.

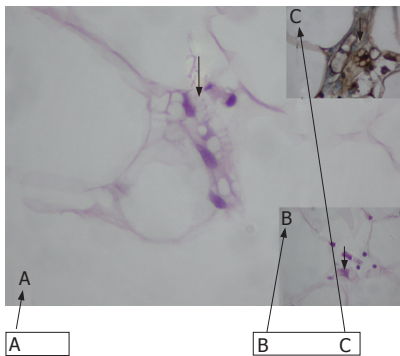


Figure 2 Multivacuolated (A) and univacuolated (B) lipoblasts as well as a multivacuolated lipoblast positive immunohistostain for S-100 protein (C).

incidence between age 50 and 65. It is the most common soft tissue sarcoma appearing anywhere in the body. Gastric liposarcomas are extremely rare and characterized by an exophytic growth being adherent to the gastric wall. Its typical location is in the lesser curvature. Gastric liposarcomas originate due to proliferation of undifferentiated mesenchymal cells within the submucosa and the tunica muscularis layer of the stomach. As with other sarcomas there are no characteristic clinical findings^[5].

Upper gastrointestinal series may support the diagnosis by the appearance of submucosal filling defects. Gastroscopy may sometimes reveal ulcerations^[5]. The definite diagnosis is best established by CT examination. There is a strong correlation between CT and histopathological findings. Cytology has a value in diagnosis since in adipose tissue tumors with fat content less than 75% of the tumor volume, liposarcoma is the most likely diagnosis^[6].

Histologically, there are four types of liposarcomas: well- differentiated, myxoid, round cell and pleomorphic. Well- differentiated liposarcomas account for 40% of all liposarcomas, having a peak incidence between the 5th and 7th decades. They are further subdivided into adipocytic,

sclerosing, inflammatory and spindle cell subtypes^[7]. Well-differentiated liposarcomas have a risk for local recurrence but no potential for metastasis. Myxoid liposarcomas are characterized by a myxoid matrix and considered to be low to intermediate grade lesions. In round cell type, there is an excessive proliferation of small rounded cells. Pleomorphic liposarcoma is a highly malignant lesion characterized by increased mitotic activity and hemorrhage as well as necrosis^[8]. The term dedifferentiated liposarcoma has been used to refer to lesions that appear to begin as low-grade lesions but progress to high grade tumors and show evidence of nonlipogenic differentiation^[9].

The relation between the histopathological type and CT findings is evident in such a way that well-differentiated liposarcomas show the classic heterogeneous density, myxoid type shows liquid cystic changes and round cell and pleomorphic types are characterized by a nonspecific solid structure^[1,5].

Differential diagnosis of gastric liposarcoma includes peritoneal liposarcoma, carcinoma engulfing perivisceral fat, gastric stromal tumors, hepatic metastases adjacent to the stomach, peritoneal carcinomatosis, lymphoma and primary tumor of the omentum^[10].

In conclusion, whenever a large exophytic mass originating from the gastric wall is revealed at computed tomography in the absence of secondary organ and peritoneal involvement. The diagnosis of a gastric liposarcoma should be considered, the treatment of choice is surgical removal.

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ACKNOWLEDGMENTS

Acknowledgments to Reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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