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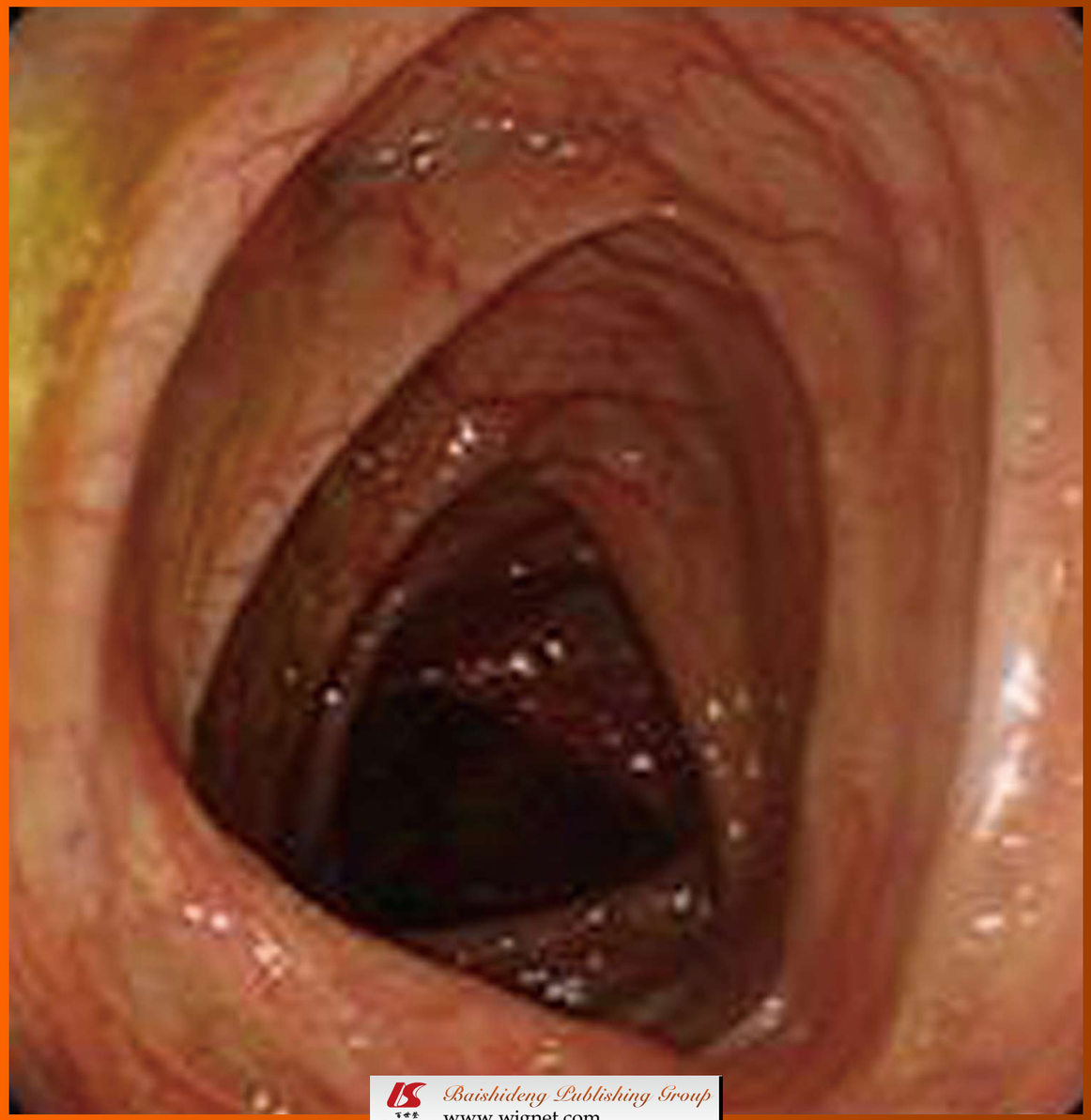

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Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

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Beijing Baishideng BioMed Scientific Co., Ltd.
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
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No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-5908-0039
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EDITORIAL

Neurogenic bowel dysfunction in patients with spinal cord injury, myelomeningocele, multiple sclerosis and Parkinson's disease

Richard A Awad

Richard A Awad, Experimental Medicine and Motility Unit, Gastroenterology Service U-107, Mexico City General Hospital, 06726 México, DF, México
Author contributions: Awad RA solely contributed to this paper.
Correspondence to: Richard A Awad, MD, MSc, Head and Professor, Experimental Medicine and Motility Unit, Gastroenterology Service U-107, Mexico City General Hospital, Dr. Balmis No. 148, Col. Doctores, 06726 México, DF, México. awadrichardalexander@prodigy.net.mx
Telephone: +52-55-50043806 Fax: +52-55-50043806
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Abstract

Exciting new features have been described concerning neurogenic bowel dysfunction, including interactions between the central nervous system, the enteric nervous system, axonal injury, neuronal loss, neurotransmission of noxious and non-noxious stimuli, and the fields of gastroenterology and neurology. Patients with spinal cord injury, myelomeningocele, multiple sclerosis and Parkinson's disease present with serious upper and lower bowel dysfunctions characterized by constipation, incontinence, gastrointestinal motor dysfunction and altered visceral sensitivity. Spinal cord injury is associated with severe autonomic dysfunction, and bowel dysfunction is a major physical and psychological burden for these patients. An adult myelomeningocele patient commonly has multiple problems reflecting the multisystemic nature of the disease. Multiple sclerosis is a neurodegenerative disorder in which axonal injury, neuronal loss, and atrophy of the central nervous system can lead to permanent neurological damage and clinical disability. Parkinson's disease is a multisystem disorder involving dopaminergic, noradrenergic, serotoninergic and cholinergic systems, characterized by motor and non-motor symptoms. Parkinson's disease affects several neuronal structures outside the substantia nigra, among which is the enteric nervous system. Recent reports have shown that the lesions in the enteric nervous system occur in very early stages of the disease, even before the involvement of the central nervous system. This has led to the postulation that the enteric nervous system could be critical in the pathophysiology of Parkinson's disease, as it could represent the point of entry for a putative environmental factor to initiate the pathological process. This review covers the data related to the etiology, epidemiology, clinical expression, pathophysiology, genetic aspects, gastrointestinal motor dysfunction, visceral sensitivity, management, prevention and prognosis of neurogenic bowel dysfunction patients with these neurological diseases. Embryological, morphological and experimental studies on animal models and humans are also taken into account.

Key words: Neurogenic bowel dysfunction; Spinal cord injury; Myelomeningocele; Multiple sclerosis; Parkinson's disease; Central nervous system; Enteric nervous system

Peer reviewer: Akio Inui, MD, PhD, Professor, Department of Behavioral Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

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INTRODUCTION

Exciting new features have been described concerning neurogenic bowel dysfunctions (NBD), including interactions between the central nervous system (CNS), enteric nervous system (ENS), neurotransmission of noxious and non-noxious stimuli, and the fields of gastroenterology and neurology. Patients with spinal cord injury (SCI), myelomeningocele (MMC), multiple sclerosis (MS) and Parkinson's disease (PD) present with autonomic dysreflexia[1], serious upper and lower NBD characterized by constipation[2], incontinence, severe gastrointestinal (GI) motor dysfunction[3] and altered visceral sensitivity[4]. SCI is associated with severe autonomic dysfunction, with bowel dysfunction as a major physical and psychological burden for these patients[5]. The outcome of MMC patients is fraught with multiple problems reflecting the multisystemic nature of the disease[6]. MS is a devastating autoimmune disease[7] with symptoms dependent on the clinical type and the site of lesions[8]. It has been considered a chronic, inflammatory disorder of the central white matter in which demyelination results in the ensuing physical disability. Recently, MS is viewed as a neurodegenerative disorder in which axonal injury, neuronal loss, and atrophy of the CNS can lead to permanent neurological and clinical disability, in which mitochondrial DNA defects are involved[9]. PD is considered as a disorder involving dopaminergic, noradrenergic, serotoninergic, and cholinergic systems, characterized by motor and non-motor symptoms[10]. Interestingly, in recent years it has become evident that PD affects several neuronal structures outside the substantia nigra, between which are the ENS. Recent reports have shown that the lesions in the ENS occur at a very early stage of the disease, even before the involvement of the CNS. This has led to the hypothesis that the ENS could be critical in the pathophysiology of PD, as it could represent a point of entry for a putative environmental factor to initiate the pathological process[11]. This review covers the data related to etiology, epidemiology, clinical aspects, pathophysiology, genetics, gastrointestinal motor dysfunction, visceral sensitivity, management, prevention, and prognosis of NBD patients with these neurological diseases. Embryologic, morphological and experimental studies on animal models and humans are also taken into account.

LITERATURE REVIEW

Search strategy

A Medline search was performed using the following subject headings: spinal cord injury, neural tube defects (NTD), myelomeningocele, multiple sclerosis, Parkinson's disease, animal models, and human. The date of the most recent search was February 28, 2011.

Selection criteria

Clinical, epidemiological, pathophysiological, motor dysfunction, visceral sensitivity and experimental studies on animal models and patients with SCI, MMC, MS, PD, as well as specific therapies for these neurological diseases involving bowel dysfunction were reviewed. Issues related to genetics, embryology, morphology, prevention and prognosis were also taken into account.

Data collection and analysis

A total of 177 articles were included in the analysis.

ETIOLOGY

SCI etiology is generally divided into traumatic and non-traumatic causes[12].

The onset of NTD occurs at 21-28 d of embryonic development[13]. MMC results from lack of closure of the neural tube during this stage[14]. Its etiology is complex, involving both genetic and environmental factors[15]. A maternal effect as well as a gender-influenced effect, have been suggested as part of its etiology[16]. Although there are more than 200 small animal models with NTD, most of them do not replicate the human disease phenotype. The candidate genes studied for risk association with spina bifida include those important in folic acid metabolism, glucose metabolism, retinoid metabolism, apoptosis, and those that regulate transcription in early embryogenesis[17].

MS is an etiologically unknown disease with no cure[7]. It is the leading cause of neurological disability in young adults, affecting over two million people worldwide. MS has been considered a chronic, inflammatory disorder of the CNS white matter in which demyelination results in the ensuing physical disability. Recently, MS has become increasingly viewed as a neurodegenerative disorder in which axonal injury, neuronal loss, and atrophy of the CNS can lead to permanent neurological damage and clinical disability[9].

GI dysmotility in PD has been attributed to the peripheral neurotoxin action[18]. Recently, it has been suggested that sporadic PD has a long prodromal period and several nonmotor features develop during this period. Hawkes *et al*[19] proposed that a neurotropic viral pathogen may enter the brain *via* nasal route with anterograde progression into the temporal lobe or *via* gastric route, secondary to the swallowing of nasal secretions. These might contain the neurotropic pathogen that, after penetration of the epithelial lining, could enter the axons of the Meissner plexus and, through transsynaptic transmission, reach the preganglionic parasympathetic motor neurons of the vagus nerve. This would allow retrograde transport into the medulla and from there into the pons and midbrain until the substantia nigra is reached[19]. A summary of suggested pathogenesis of GI disorders underlying PD is shown in Table 1.

EPIDEMIOLOGY

Traumatic SCI represents a significant public health problem

Table 1 Suggested pathogenesis of gastrointestinal disorders underlying Parkinson's disease

GI pathogenesis	Disorder
Peripheral neurotoxine action	Interstitial cells of Cajal involvement[18]
GI flora? Neurotropic viral pathogen	GI disorders[19]
GI flora? *Helicobacter pylori*	Modified l-dopa pharmacokinetics[102]
GI dysmotility: Early lesions in the enteric nervous system	GI dysfunction[11,163]
GI dysmotility: Disruption in parts of the CNS	Neurogenic dysphagia[54]
GI dysmotility: Lewy bodies in esophageal myenteric plexuses	Manometric abnormalities[97,98]
GI dysmotility: Reduction amplitude of peristaltic contractions	Decreased gastric motility[105]
GI dysmotility: Gastric pacemaker disturbances	Gastric dysrhythmias[106]
GI dysmotility: Loss of enteric dopaminergic neurons	Changes in colon motility[173]
Neurotransmitter dysfunction: Altered enteric nitrergic systems	Disturbed distal gut transit[95]
Neurohormone involvement: Neurotensin	GI disorders[103]
Levodopa	Altered oral phase of deglutition[96]
Monoamine dysfunction	Nonmotor symptoms[176]

GI: Gastrointestinal; CNS: Central nervous system.

worldwide[20]. Each year, 11 000 individuals are estimated to have SCI in the United States[21] with a mortality rate of 27.4 per million people. An annual incidence of 33.6 per million is reported in Greece and 19.5 per million in Sweden[22], while in Denmark the number of SCI patients is about 3000.

NTD is the second most common birth defect, with an incidence of 1/1000. MMC is the most common subtype (66.9%)[16]. NTD is rarely reported in black Americans and Japanese, but is not so rare in Cameroon and sub-Saharan black Africans, with an incidence of 1.9 cases per 1000 births[23]. In Switzerland, the incidence of NTD in children is 0.13 per thousand, corresponding to 9-10 affected newborns each year[15], while in Thailand, the incidence is 0.67 per 1000 births[24]. NTD is reported in adolescents aged 15-18 years[25] and in young adults aged 20-23 years[26].

MS affects young and middle-aged people[27], the mean age at disease onset is 30.7 ± 6.4 years, and it is believed that pregnancy, postpartum status and vaccines[8], as well as infection with Epstein-Barr virus[28], may influence the onset and course of the disease. An increase in females and an almost universal increase in the prevalence and incidence have been reported, challenging the theory of a geographical gradient of incidence in Europe and North America[29]. It affects 100 000 people in the United Kingdom[30], with a prevalence of 30.9/100 000 in Herzegovina[31]. An association between the risk of MS and the season of birth suggested that decreased exposure to the sunshine in the winter leading to low vitamin D levels during pregnancy is an area that needs further research[32].

PD is the second most common neurodegenerative disease after Alzheimer's disease[11], affecting one million people in the United States each year[33], and 20% of the population aged > 65 years in Mexico[34]. It is described in sporadic and familial forms[35] (at least 2 individuals are affected within 2-3 consecutive generations of a family).

DIAGNOSIS, CLINICAL DATA AND SYMPTOMS

Neurophysiologic testing of the sacral reflex is useful in the diagnosis of sacral lower motor neuron lesions, and increased elicitability of the penilo-cavernosus reflex is reported in patients with chronic SCI[36]. Patients with SCI may present[4] with brain anatomical changes of loss of motor control, chronic neuropathic[37] and abdominal pain[38], urinary[39]and sexual dysfunction[40], decubiti[41], neurogenic immune depression syndrome[42], and an increased risk of having a depressive disorder[43]. Spinal cord lesions affect colorectal motility, anorectal sensation, anal sphincter function, and cause neurogenic constipation[44]. Defecation is abnormal in 68% of cases, digital stimulation is required by 20%, suppositories by 10% and enemas by 28% of cases. Time spent in each defecation is more than 30 min in 24% cases. In children aged four years or older, daily fecal incontinence occurred in 14% and weekly incontinence in 14% cases[45]. SCI patients usually do not perceive the normal desire for defecation, rather describing it as abdominal distension, hardened or cool abdomen, hardening of the legs, abdominal pain, chills and dizziness, itching of the head, and a feeling of pain at the sacrum level[4]. Additionally, SCI subjects may develop autonomic dysreflexia in response to noxious stimulus[46]. Cardiovascular dysregulation, characterized by paroxysmal high blood pressure episodes, is the most prominent feature and is precipitated by manual emptying of rectal contents and by gastric and bowel distension[47]. Regarding the gravity of this issue, an NBD score (0-6 very minor, 7-9 minor, 10-13 moderate and 14 severe)[48], an international bowel function basic[49] and extended[50] SCI data set, as well as an international standard to document the remaining autonomic function after SCI[40] have been developed.

Prenatal screening with α-fetoprotein and ultrasonography have allowed the prenatal diagnosis of NTD in current obstetric care[51]. In an animal model with naturally occurring spina bifida (curly tail/loop tail mouse), using standard enzyme linked immunosorbent assay techniques, detection of amniotic fluid levels of the neurofilament heavy chain, glial acidic fibrillary protein and S100B, seems to provide important information for balancing the risks and benefits, both to mother and child, of in utero

surgery for MMC[52]. Colorectal problems are common in children with MMC and their impact on the quality of life becomes more severe as the child grows up.

Diagnosis of MS is made according to the McDonald and the Poser criteria, with the McDonald criteria showing a higher sensitivity for diagnosis[53]. Bowel symptoms are reported to be common in MS, including constipation (29%-43%) and fecal incontinence (over 50%), and 34% of patients spending more than 30 min a day managing their bowel movement[30]. Neurogenic dysphagia is also present[54]. Autonomic dysreflexia may occur in MS[55], characterized by hypertensive attacks, palpitations, difficulty in breathing, headaches and flushing[56]. Autonomic symptoms are disorders of micturition, impotence, sudomotor and GI disturbances, orthostatic intolerance as well as sleep disorders[57]. Neuropsychiatric symptoms include abnormalities in cognition, mood and behavior (major depression, fatigue, bipolar disorder, euphoria, pathological laughing and crying, anxiety, psychosis and personality changes). Major depression is a common neuropsychiatric disorder, with an approximate 50% lifetime prevalence rate[58]. Pediatric MS has been identified as an important childhood acquired neurologic disease[59].

GI diagnosis in PD[60] includes history, clinical examination, barium meal, breath test, stomach scintigraphy and colonic transit time[61]. Oropharyngeal dysphagia is recognized by difficulty in transferring a food bolus from the mouth to the esophagus or by signs and symptoms of aspiration pneumonia or nasal regurgitation[62]. PD is actually considered a neurodegenerative process that affects several neuronal structures outside the substantia nigra. Reports have shown that the lesions in the ENS occurred at a very early stage of the disease, even before CNS involvement[11]. GI symptoms are very important, as GI diseases may also display neurological dysfunction as part of their clinical picture[63]. PD patients have motor and non-motor fluctuations classified into three groups: autonomic, psychiatric, and sensory[64]. GI dysfunction is the most common non-motor symptom which comprises sialorrhea, swallowing disorders[65], dysphagia[66], acid regurgitation, pyrosis[67], early satiety, weight loss, constipation[68], incomplete rectal emptying, the need for assisted defecation and an increased need for oral laxatives[69].

PATHOPHYSIOLOGY

Genetic factors

Data was obtained from 1066 NTD families, 66.9% with MMC, suggesting a maternal effect, as well as a gender-influenced effect in the etiology of NTD[16]. Telomerase, the reverse transcriptase that maintains telomere DNA, is important for neural tube development and bilateral symmetry of the brain. However, it is reported that variants in the telomerase RNA component (TERC) are unlikely to be a major risk factor for the most common form of human NTD, lumbosacral MMC[70].

The association between a polymorphism in the *ABCB1* gene and PD has been observed. The ATP-binding cassette, sub-family B, member 1 (*ABCB1*) gene encoding P-glycoprotein (P-gp), has been implicated in the pathophysiology of PD due to its role in regulating the transport of endogenous molecules and exogenous toxins. ABCB1 polymorphisms thus constitute an example of how genetic predisposition and environmental influences may combine to increase the risk of PD[71]. On the other hand, extensive ENS abnormalities in mice transgenic for PD-associated α-synuclein gene mutations precede CNS changes. Most PD is sporadic and of unknown etiology, but a fraction is familial. Among familial forms of PD, a small portion is caused by missense (A53T, A30P and E46K) and copy number mutations in SNCA, which encodes α-synuclein, a primary protein constituent of Lewy bodies, the pathognomonic protein aggregates found in neurons in PD[72].

Gastrointestinal motor dysfunction and visceral sensitivity

Fecal incontinence in SCI, MMC and MS is mainly due to abnormal rectosigmoid compliance and recto-anal reflexes, loss of recto-anal sensitivity and loss of voluntary control of the external anal sphincter[73]. On the other hand, constipation is probably due to immobilization, abnormal colonic contractility, tone and recto-anal reflexes, or side effects from medication. SCI patients have a higher incidence of esophagitis and esophageal motor abnormalities[74], gastric stasis, paralytic ileus, abdominal distension[75], partial or complete loss of the sensations upon defecation, constipation[75], hemorrhoids[76], and need for assisted digital evacuation than controls[75]. Studies have shown a range of neurological alterations, such as low amplitude, slowly propagating abnormal peristaltic esophageal contraction[74], a decrease in phase Ⅲ of the interdigestive motor complex[77], reduction in gastric emptying[78], delayed GI transit, higher colonic myoelectric activity, reduced emptying of the left colon, and a suboptimal postprandial colonic response[79]. Visceral sensitivity testing according to Wietek *et al*[80] may be a future requirement, in addition to the American Spinal Injury Association (ASIA) criteria, in the assessment of the completeness of cord lesions in patients diagnosed with complete spinal cord transection, as some report the sensation of distension of the rectum. In our laboratory, with barostat methodology, we found that complete supraconal SCI patients preserve rectal sensation, and present with impaired rectal tone and impaired response to food. This data supports the fact that barostat sensitivity studies can complement ASIA criteria to confirm a complete injury. Our results also suggest that intact neural transmission between the spinal cord and higher centers is essential for noxious stimulus, but not for non-noxious stimuli, that patients with supraconal lesions may present PP visceral hypersensitivity, and that incontinence and constipation may not be related solely to continuity of the spinal cord[4,81]. Suttor *et al*[82], using

Table 2 Suggested pathogenesis of gastrointestinal disorders underlying spinal cord injury, myelomeningocele and multiple sclerosis

Disease	GI pathogenesis	Disorder
Spinal cord injury Myelomeningocele Multiple sclerosis	Abnormal rectosigmoid compliance Loss of recto-anal sensitivity Loss of voluntary control of the external anal sphincter	Fecal incontinence[73]
Spinal cord injury Myelomeningocele Multiple sclerosis	Immobilization, abnormal colonic contractility, side effects of medication	Constipation[94]
Multiple sclerosis	Paradoxical puborectalis contraction	Constipation[94]
Multiple sclerosis	Bladder distension	Autonomic dysreflexia[56]
Myelomeningocele	Severe constipation	Ventriculoperitoneal shunt malfunction[87]
Myelomeningocele	Visceral hypersensitivity	Constipation and impaired rectal tone and response to food[88]
Myelomeningocele	Higher spinal level of cord lesion, completeness of cord injury and longer duration of injury	Severe neurogenic bowel dysfunction[20]
Spinal cord injury	Noxious stimulus	Autonomic dysreflexia[46]
Spinal cord injury	Manual emptying of rectal contents and gastric and bowel distension	Cardiovascular dysregulation[47]

a dual barostat in six cervical SCI patients without NBD, reported that intact neural transmission between the spinal cord and higher centres is not essential for normal colorectal motor response from feeding to distension. Lumbosacral neuropathy was demonstrated in 90% of SCI subjects[83] using translumbar and trans-sacral motor-evoked potentials.

In MMC, studies have revealed swallowing disorders characterized by difficulty in bolus formation, nasopharyngeal and gastroesophageal reflux, tracheobronchial aspiration, and vocal cord paralysis[84], as well as a longer mean colonic transit time not related to the level of the spinal lesion[85] and reduction in anal sphincter pressure[86]. Ventriculoperitoneal shunt malfunction may occur in patients with MMC, and severe constipation that increases intra-abdominal pressure resulting in raised intracranial pressure, seems to be one of the causes[87]. Visceral sensitivity studies with the barostat reveal that constipated children with MMC present with impaired rectal tone, impaired response to food and postprandial visceral hypersensitivity[88].

GI dysfunction occurs in MS as in other neurologic diseases[63]. Slow gastric emptying rate[89], increased colonic transit time[90], absent PP colonic motor and myoelectric responses[91], altered maximal contraction pressures and anal inhibitory reflex threshold[92], impaired function of the external anal sphincter, and increased thresholds of conscious rectal sensation[93] have been reported. Paradoxical puborectalis contraction is common in MS patients with constipation[94] and it seems that autonomic dysreflexia occurs due to bladder distension[56]. A summary of suggested pathogenesis of GI disorders underlying spinal cord injury, myelomeningocele, and multiple sclerosis is shown in Table 2.

In PD, dysphagia, impaired gastric emptying and constipation may precede its clinical diagnosis for years[61]. ENS involvement could be critical as it may represent a point of entry for a putative environmental factor to initiate the pathological process[11]. On the other hand, the mechanisms related to enteric autonomic dysfunctions may involve the enteric dopaminergic or nitrergic systems. It has been reported that rats with a unilateral 6-hydroxydopamine lesion of nigrostriatal dopaminergic neurons develop marked inhibition of propulsive activity compared with sham-operated controls. Results suggest that disturbed distal intestinal transit may occur as a consequence of reduced propulsive motility, probably due to an impairment of a nitric oxide-mediated descending inhibition during peristalsis[95]. Neurogenic dysphagia may also appear in PD. It may be caused by a disruption in different parts of the CNS (supranuclear level, level of motor and sensory nuclei taking part in the swallowing process and peripheral nerve level) or a neuromuscular disorder[54]. It is also suggested that levodopa plays a role in the oral phase of deglution in PD[96]. Dysphagia is present in up to 50% of PD cases and seems to be correlated with manometric irregularities[97,98]. Castell *et al*[97] have described esophageal manometric abnormalities in 73% of PD patients characterized by complete aperistalsis or multiple simultaneous contractions (diffuse esophageal spasm) of the distal esophagus. They also reported repetitive proximal esophageal contractions[99], a very interesting finding supporting a previous report of a link between PD, achalasia[100], and scleroderma (e.g., PD and achalasia have Lewy bodies in the esophageal myenteric plexuses and the substantia nigra, as well as evidence of degeneration of the dorsal motor nucleus of the vagus), and esophageal manometric abnormalities were found in these three diseases. A link between PD and *Helicobacter pylori* (*H. pylori*)[101] has also been described, where HP eradication may improve the clinical status of infected patients with PD and motor fluctuations by modifying l-dopa pharmacokinetics[102]. Neurotensin, a 13 amino acid neurohormone located in the synaptic vesicles and released from the neuronal terminals in a calcium-dependent manner, is involved in the pathophysiology of PD and other neurodegenerative conditions[103]. Constipation and gastric atony are important

non-motor symptoms[104]. There is a trend toward a decreased gastric motility in PD patients as compared with healthy controls due mainly to a significant reduction in the amplitude of peristaltic contractions[105]; other authors have found gastric dysrhythmias indicating gastric pacemaker disturbances[106]. Slow transit in the colon has been reported[107], and using ano-rectal manometry, decreased basal anal sphincter pressures, prominent phasic fluctuations on squeeze pressure, and a hyper-contractile external sphincter response to the rectosphincteric reflex have been documented. It has also been suggested that dystonia of the external anal sphincter causes difficult rectal evacuation and the loss of dopaminergic neurons in the ENS may lead to slow-transit constipation[73].

MANAGEMENT

Managing SCI bowel function is complex, time consuming and remains conservative[75]. The use of manual evacuation[108], treatment with oral laxatives[108] and abdominal massage[109] have all been reported. Transanal irrigation is reported safe and can be used in most patients suffering from NBD[110], its results represent a lower total cost than conservative bowel management[111]; however, its rate of success is only 35% after 3 years[110]. Recent approaches include sacral neuromodulation[112] and dorsal penile/clitoral nerve neuromodulation for the treatment of constipation, as well as magnetic stimulation for NBD treatment[113]. Other options include colostomy, ileostomy, malone anterograde continence enema, and sacral anterior root stimulator implantation[114]. However, good quality research data is needed to evaluate the effects of these treatments for this condition.

For MMC patients with constipation, polyethylene glycol[44,115] and the use of transanal irrigation[116] seem to be effective, however, a majority of children found the procedure time consuming and did not help them to achieve independence at the toilet[117]. For incontinence, the approaches included intravesical[118] and transrectal electro-stimulation[119]; nevertheless these procedures lack well-designed controlled trials. For constipation and incontinence, biofeedback is used[120]. Surgical closure of MMC is usually performed in the early postnatal period, however, not all patients benefit from fetal surgery in the same way[121]. The management of cervical MMC is early surgical treatment with microneurosurgical techniques. Surgical excision of the lesions with intradural exploration of the sac to release any potential adhesion bands is safe and effective[122].

The current therapies for MS are few, symptom-related, and experimental[7]. In patients seen due to constipation, incontinence, or a combination of these symptoms a beneficial effect of biofeedback was attributed to some but not to all patients[123]. Other approaches include oral administration of probiotic bacteria, *Lactobacillus casei* and *Bifidobacterium breve*, which do not seem to exacerbate neurological symptoms[124]. An overactive bladder is successfully treated in 51% of cases with anticholinergic medication[125]. The use of agonists or antagonists of prostaglandin-receptors may be considered as a new therapeutic protocol in MS. The reason is that prostaglandins as arachidonic acid-derived autacoids play a role in the modulation of many physiological systems including the CNS, and its production is associated with inflammation, which is a feature in MS[126].

Levodopa, a prodrug of dopamine, is one of the main treatment options in PD[127]. However, in contrast to motor disorders, pelvic autonomic dysfunction is often refractory to levodopa treatment[128]. One point to bear in mind is that treatments should facilitate intestinal absorption of levodopa[128]. Current levodopa products are formulated with aromatic amino acid decarboxylase inhibitors such as carbidopa or benserazide to prevent the metabolism of levodopa in the GI tract and systemic circulation[127]. Food appears to affect the absorption of levodopa, but its effects vary with formulations and studies suggest that a high protein diet may compete with the uptake of levodopa into the brain, thus resulting in reduced levodopa effects[127]. Regarding disturbed motility of the upper GI-tract, hypersalivation is reported to be reduced by anticholinergics or botulinum toxin injections[61] while therapy for dysphagia includes rehabilitative, surgical, and pharmacologic treatments[129]. Regarding constipation, tegaserod improves both bowel movement frequency and stool consistency[130]. Mosapride citrate, a 5-HT4 agonist and partial 5-HT3 antagonist, in contrast to cisapride, does not block K (+) channels or D2 dopaminergic receptors[131]. Other prokinetics agents include metoclopramide, domperidone, trimebutine, cisapride, prucalopride, and itopride[132]. Polyethylene glycol[61], functional magnetic stimulation[133], and psyllium are also used[134]. However, the clinical significance of any of these results is difficult to interpret and it is not possible to draw any recommendation for bowel care from published trials, until well-designed controlled trials with adequate numbers of patients and clinically relevant outcome measures become available[134]. Recently, stem cells have been used as an alternate source of biological material for neural transplantation to treat PD. The potential benefits for this are relief of parkinsonian symptoms and a reduction in the doses of parkinsonian drugs employed. However, the potential risks include tumor formation, inappropriate stem cell migration, immune rejection of transplanted stem cells, hemorrhage during neurosurgery and postoperative infection[135].

PREVENTION AND PREDICTORS

An analysis of predictors of severe NBD in SCI shows that those with a cervical injury or a thoracic injury had a higher risk of severe NBD than those with a lumbar spine injury. Also those classified as ASIA a had a 12.8-fold higher risk of severe NBD than persons with ASIA D. Besides, a longer duration of injury (≥ 10 years) was considered as another risk factor of severe NBD. Moderate-to-severe depression was associated with reduced

bowel function. The results showed that a higher spinal level of cord lesion, completeness of cord injury and a longer duration of injury (≥ 10 years) could predict the severity of NBD in patients with SCI[20]. It is reported that clinical variables are not the best predictors of long-term mortality in SCI. Instead, the significant effect of poor social participation and functional limitations seem to persist after adjustment for other variables[136].

Folic acid supplementation reduced the incidence of NTD in several geographical regions. However, the incidence is still high and associated with a serious morbidity[137]. A study done in newborn babies with NTD and their mothers revealed an association between NTD and decreased hair zinc levels, so large population-based studies are recommended to confirm the association between zinc and NTD[138]. The prevalence of scoliosis in patients with MMC has been reported to be as high as 80%-90%. A study aiming to determine clinical and radiographic predictors of scoliosis in patients with MMC reported that the clinical motor level, ambulatory status, and the level of the last intact laminar arch are predictive factors for the development of scoliosis. It is suggested that in patients with MMC, the term scoliosis should be reserved for curves of > 20 degrees, it is also noteworthy that new curves may continue to develop until the age of fifteen years[139]. Other authors attempting to obtain a spine deformity predictor based on a neurological classification performed at five years of age report that group I (L5 or below) is a predictor for the absence of spinal deformity, group Ⅲ (L1-L2) or Ⅳ (T12 and above) is a predictor for spinal deformity and group Ⅳ is a predictor of kyphosis. This data confirms that future spinal disorders are expected in some patients, while no spinal deformity is expected in others[140]. Other reports indicate that the horizontal sacrum is an indicator of the tethered spinal cord in spina bifida aperta and occulta, as signs and symptoms indicative of a tethered spinal cord appear to correspond to increases in the lumbosacral angle[141]. It is also reported that behavior regulation problems in children with MMC are predicted by parent psychological distress, and that more shunt-related surgeries and a history of seizures predict poorer metacognitive abilities[142]. It seems that adults with MMC and shunted hydrocephalus may be at risk for decreased survival[143].

Inadequate serum vitamin D concentrations are associated with complications of some health problems including MS, which support a possible role for vitamin D supplementation as an adjuvant therapy[144]. In addition, it has been suggested that the favorable effect of sunlight ascribed to an increased synthesis of vitamin D may prevent certain autoimmune diseases, particularly MS. For this reason, limited sunbathing should be publically encouraged[145]. It has also been suggested that altering the composition of the gut flora may affect susceptibility to experimental autoimmune encephalomyelitis, an animal model of MS[146]. This data could have significant implications for the prevention and treatment of autoimmune diseases. In relation to this, an interesting new proposal shows that the GI tract is a vulnerable area through which pathogens (such as *H. pylori*) may influence the brain and induce MS, mainly *via* fast axonal transport by the afferent neurons connecting the GI tract to brain[147].

Symptoms such as dysphagia, impaired gastric emptying and constipation may precede the clinical diagnosis of PD by years and, in the future, these symptoms might serve as useful early indicators of the premotor stage[61]. Motor handicaps, such as rigor and action tremor, are independent predictors of solid gastric emptying[148]. It is currently recommended that the approach to PD should include strategies for detecting the disease earlier in its course and, eventually, intervening when the disease is in its nascent stage. The term Parkinson's associated risk syndrome has been coined to describe patients at risk for developing PD. These patients may have genetic risk factors or may have subtle, early non-motor symptoms including abnormalities in olfaction, GI function, cardiac imaging, vision, behavior, and cognition[149].

EMBRYOLOGICAL, MORPHOLOGICAL AND EXPERIMENTAL STUDIES AND ANIMAL MODELS

Embryology and morphology

Considerable insight into both normal neural tube closure and the factors possibly disrupting this process has been reported in recent years, yet, the mechanisms by which NTD arises as well as its embryogenesis remain elusive[150]. Normal brain development throughout childhood and adolescence is characterized by decreased cortical thickness in the frontal regions and region-specific patterns of increased white matter myelination and volume. Subjects with MMC show reduced white matter and increased neocortical thickness in the frontal regions, suggesting that spina bifida may reflect a long-term disruption of brain development that extends far beyond the NTD in the first week of gestation[151]. These variations in the diffusion metrics in MMC children are suggestive of abnormal white matter development and persistent degeneration with advancing age[152].

In rat fetuses with retinoic acid induced MMC, the normal smooth muscle and myenteric plexus development of the rectum and normal innervations of the anal sphincters and pelvic floor suggest that MMC is not associated with a global neuromuscular alteration in development of lower GI structures[153]. Besides, fetal surgery for repair of MMC allows normal development of anal sphincter muscles in sheep. Histopathologically, in the external sphincter muscles, the muscle fibers were dense, while in the internal sphincter muscles, endomysial spaces were small, myofibrils were numerous, and fascicular units were larger than those in unrepaired fetal sheep[154]. Studies in the development of the pelvic floor muscles of murine embryos with anorectal malformations, demonstrate that the embryos show an impaired

anatomic framework of the pelvis possibly caused by neural anomalous development, whereas muscle development proceeded physiologically. These results support the hypothesis that pelvic floor muscles may function in children with anorectal malformations, in whom neural abnormalities such as MMC have been ruled out, if the surgical correction is appropriately completed[155]. A mouse model was reported about the sharing of the same embryogenic pathway in anorectal malformations and anterior sacral MMC formation[156]. Indeed, some of the brain malformations associated with MMC in human patients are also found in the uncorrected fetal lamb model of MMC[157]. The late stage of gestation is important due to the presence of morphological changes. A study of in-utero topographic analysis of astrocytes and neuronal cells in the spinal cord of mutant mice with MMC revealed that at day 16.5 of gestation there is a deterioration of neural tissue in MMC fetuses, mainly in the posterior region, progressing until the end of gestation with a marked loss of neurons in the entire MMC placode. This study delineated the quantitative changes in astrocytes and neurons associated with MMC development during the late stages of gestation[158]. Data supported by other investigators show, in Curly tail/loop tail mouse fetuses, that around birth the unprotected neural tissue is progressively destroyed[159].

Traditionally, PD is attributed to the loss of mesencephalic dopamine-containing neurons; nonetheless, additional nuclei, such as the dorsal motor nucleus of the vagus nerve and specific central noradrenergic nuclei, are now identified as targets of PD[160]. Early in 1988, Wakabayashi[161] described the presence of Lewy bodies in Auerbach's and Meissner's plexuses of the lower esophagus, indicating that these are also involved in PD. Later on, the presence of α-synuclein immunoreactive inclusions in neurons of the submucosal Meissner plexus, whose axons project into the gastric mucosa and terminate in direct proximity to fundic glands, was reported[162]. The authors propose that these elements could provide the first link in an uninterrupted series of susceptible neurons that extend from the enteric tract to the CNS. The existence of such an unbroken neuronal chain lends support to the hypothesis that a putative environmental pathogen capable of passing the gastric epithelial lining might induce α-synuclein misfolding and aggregation in specific cell types of the submucosal plexus and reach the brain *via* a consecutive series of projection neurons. A recent study aimed at characterizing the neurochemical coding of the ENS in the colon of a monkey model of PD, showed that this element induces major changes in the myenteric plexus and to a lesser extent in the submucosal plexus of monkeys. This data reinforces the observation that lesions of the ENS occur in the course of PD and that this might be related to the GI dysfunction observed in this pathology[163].

Experimental approaches and animal models

Animal models used in MMC include an ovine model constituted by fetal lambs[164], fetal sheep[165], a Macaca mulatta model[166], a mice model[158], and a fetal rabbit model[167]. Several experimental approaches have been used. To study the correction of a MMC-like defect in pregnant rabbits, a spinal defect was surgically created in some of their fetuses at 23 d of gestation. The spinal defect was successfully repaired, and the fetal rabbit model was established for the study of intrauterine correction of an MMC-like defect[167]. A new gasless fetoscopic surgery for the correction of a MMC-like defect in fetal sheep served as an alternative to current techniques used for fetal endoscopic surgery[165]. A Macaca mulatta model was used for replicating MMC and to evaluate options for prenatal management, such as the collocation of an impermeable silicone mesh which protects the spine from amniotic liquid with results similar to skin closure[166]. In-utero analyses of astrocytes and neuronal cells in the spinal cord of mutant mice with MMC using the curly tail/loop-tail mice model have been reported. At day 16.5 of gestation, a deterioration of neural tissue in MMC fetuses was observed mainly in the posterior region, progressing until the end of gestation with a marked loss of neurons in the entire MMC placode. These results support the current concept of placode protection through in-utero surgery for fetuses with MMC[158]. Recently, the notion of prenatal neural stem cell delivery to the spinal cord as an adjuvant to fetal repair of spina bifida has been proposed[164].

The main animal model in MS was developed in mice and is called experimental autoimmune encephalomyelitis[7]. In this experimental model, it was reported that gut flora may influence the development of experimental autoimmune encephalomyelitis[146], and that despite reported blood-brain barrier disruption, CNS penetration for small molecule therapeutics does not increase in MS-related animal models[168]. The migratory potential, the differentiation pattern and long-term survival of neural precursor cells in this experimental autoimmune encephalomyelitis mice model were investigated. The results suggest that inflammation triggers migration whereas the anti-inflammatory component is a prerequisite for neural precursor cells to follow glial differentiation into myelinating oligodendrocytes[169]. A new exciting finding with this model is that a novel regulator of leukocyte transmigration into the CNS, denominated extracellular matrix metalloproteinase inducer (EMMPRIN), indeed regulates leukocyte trafficking through increasing matrix metalloproteinase activity. Amelioration of the clinical signs of experimental autoimmune encephalomyelitis by anti-EMMPRIN antibodies was critically dependent on its administration around the period of onset of clinical signs, which is typically associated with significant influx of leukocytes into the CNS. These results identify EMMPRIN as a novel therapeutic target in MS[170].

Several experimental approaches in PD deal with GI issues using diverse animal models as rats, mice and primates. The advent of transgenic technologies has contributed to the development of several new mouse models, many of which recapitulate some aspects of the

disease; however, no model has been demonstrated to faithfully reproduce the full constellation of symptoms seen in human PD[171]. As GI dysmotility in PD has been attributed in part to peripheral neurotoxin action, rats with salsolinol induced PD were studied to evaluate its effects on intramuscular interstitial cells of Cajal, duodenal myoelectrical activity and vagal afferent activity. The results suggest a direct effect of salsolinol on both interstitial cells of Cajal and the neuronal pathways for gastro-duodenal reflexes[18]. Delayed gastric emptying and ENS dysfunction in the rotenone model of PD suggested that enteric inhibitory neurons may be particularly vulnerable to the effects of mitochondrial inhibition by Parkinsonian neurotoxins and provide evidence that Parkinsonian GI abnormalities can be modeled in rodents[68]. Studies assessing the responses of myenteric neurons to structural and functional damage by neurotoxins *in vitro* reveal that neural responses to toxic factors are initially unique but then converge into robust axonal regeneration, whereas neurotransmitter release is both vulnerable to damage and slow to recover[172]. The prototypical parkinsonian neurotoxin, MPTP, as a selective dopamine neuron toxin in ENS and used in a mouse model, shows loss of enteric dopaminergic neurons and changes in colon motility[173] and its use in a primate animal model reveals changes in the myenteric plexus and, to a lesser extent, in the submucosal plexus. These models further reinforces the observation that lesions of the ENS occur in the course of PD which might be related to GI dysfunction observed in this pathology[163]. In order to determine the changes in the dopaminergic system in the GI tract, two kinds of rodent models were used. In one, 6-hydroxydopamine was microinjected into the bilateral substantia nigra of a rat. In the other, MPTP was injected intraperitoneally into mice. The results suggest that the different alterations of dopaminergic system observed in the GI tract of the two kinds of PD models might underline differences in GI symptoms in PD patients and might be correlated with the disease severity and disease process[174]. In a similar rat model, it is reported that a unilateral 6-hydroxydopamine lesion of nigrostriatal dopaminergic neurons led to a marked inhibition of propulsive activity compared with sham-operated controls, suggesting that disturbed distal gut transit, reminiscent of constipation in the clinical setting, may occur as a consequence of reduced propulsive motility, likely due to an impairment of nitric oxide-mediated descending inhibition during peristalsis[95]. Observations in Parkinsonian primates showed that when the implanted undifferentiated human neural stem cells survived, they had a functional impact as assessed quantitatively by behavioral improvement in this dopamine-deficit model[175]. Nonmotor symptoms of PD studied in an animal model with reduced monoamine storage capacity suggests that monoamine dysfunction may contribute to many of the nonmotor symptoms of PD, and interventions aimed at restoring monoamine function may be beneficial in treating the disease[176]. In a clinical approach, it was demonstrated that delay in gastric emptying did not differ between untreated, early-stage and treated, advanced-stage PD patients, suggesting that delayed gastric emptying may be a marker of the pre-clinical stage of PD[177].

CONCLUSION

This article reviews the current knowledge in all the fields of the neurological diseases with neurogenic bowel dysfunction, and the common issues in need of clarification. The hope is that with a full perspective of the situation, researchers can generate new ideas that can be useful for prevention, cure, or at least for the mean time, a better quality of life for the patient.

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EDITORIAL

Prevention of peritoneal adhesions: A promising role for gene therapy

Hussein M Atta

Hussein M Atta, Department of Surgery, Faculty of Medicine, Minia University, El-Minia 61519, Egypt
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Supported by The United States-Egypt Science and Technology Joint Fund in cooperation with United States Department of Agriculture and the Egyptian Science and Technology Development Fund under Project 739
Correspondence to: Hussein M Atta, MD, PhD, Professor, Department of Surgery, Faculty of Medicine, Minia University, Misr-Aswan Road, El-Minia 61519, Egypt. attahm@ems.org.eg
Telephone: +20-1-001407222 Fax: +20-2-22917077
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Abstract

Adhesions are the most frequent complication of abdominopelvic surgery, yet the extent of the problem, and its serious consequences, has not been adequately recognized. Adhesions evolved as a life-saving mechanism to limit the spread of intraperitoneal inflammatory conditions. Three different pathophysiological mechanisms can independently trigger adhesion formation. Mesothelial cell injury and loss during operations, tissue hypoxia and inflammation each promotes adhesion formation separately, and potentiate the effect of each other. Studies have repeatedly demonstrated that interruption of a single pathway does not completely prevent adhesion formation. This review summarizes the pathogenesis of adhesion formation and the results of single gene therapy interventions. It explores the promising role of combinatorial gene therapy and vector modifications for the prevention of adhesion formation in order to stimulate new ideas and encourage rapid advancements in this field.

Key words: Peritoneal adhesions; Tissue plasminogen activator; Gene therapy; Plasminogen activator inhibitor; Tissue inhibitor of metalloproteinase; Transforming growth factor β

Peer reviewer: Michael E Zenilman, MD, Clarence and Mary Dennis Professor and Chairman, Department of Surgery, SUNY Downstate Medical Center, PO Box 40, 450 Clarkson Avenue, Brooklyn, NY 11202, United States

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INTRODUCTION

Peritoneal adhesions are abnormal deposits of fibrous tissue that occur in the peritoneal cavity as a result of surgery or peritonitis, or their combination. Adhesions occur in more than 90% of patients following abdominal or pelvic surgery[1-3], and are only moderately reduced after laparoscopic surgical procedures compared to open surgery[4-8]. Adhesion reformation occurs postoperatively in 85% of patients, regardless of whether the adhesiolysis is performed *via* laparotomy or laparoscopy[9].

Intraperitoneal adhesions are a major source of morbidity, being the commonest cause of intestinal obstruction[10,11], secondary female infertility, and ectopic gestation[12,13]. They may also cause chronic abdominal and pelvic pain[14,15]. Small bowel obstruction is the most serious consequence of intra-abdominal adhesions. Retrospective studies have shown that 32%-75% of patients who require abdominal re-operation have adhesion-related intestinal obstruction[16,17].

Adhesions result in a large surgical workload and cost to health care systems. An epidemiological study in the United States showed that 282 000 hospital admissions in 1988 were due to adhesion-related disorders, and the cost

of in-patient adhesiolysis was $1.18 billion[18]. In 1994, 1% of all United States admissions involved adhesiolysis treatment, resulting in $1.33 billion in health care expenditure[19].

Adhesions and their associated complications are of rising medico-legal interest. Physicians worldwide need to be aware of the increasing burden of medico-legal claims arising from the complications of intra-abdominal adhesions. Successful medico-legal claims include cases of bowel perforation after laparoscopic division of adhesions, delays in the diagnosis of adhesion obstruction of the small bowel, infertility as a result of adhesions, and pain[20].

Currently, there is no effective method for preventing adhesion formation or reformation[21]. A better understanding of the pathogenesis of adhesion formation at the cellular and molecular level would undoubtedly help to develop more effective treatment strategies[3].

PATHOGENESIS

Vicious triad of trauma, hypoxia, and inflammation

The peritoneum is lined by mesothelial cells loosely attached to the basement membrane, which can readily be detached by the slightest trauma[22]. After injury to the peritoneum, a local inflammatory reaction causes increased vascular permeability in blood vessels supplying the damaged area, followed by an exudation of serosanguinous fluid rich in fibrin and inflammatory cells, ultimately leading to the formation of a fibrin matrix. Normally, the plasminogen activator activity (PAA), which resides in the mesothelial cells and submesothelial fibroblasts, degrades the fibrinous mass, resulting in healing of peritoneal surfaces (within three to five days) without adhesions. However, if the level of PAA is diminished, the fibrinous mass persists and the underlying fibroblasts migrate into the fibrinous mass. The fibroblasts then deposit extracellular matrix, including collagen and fibronectin, leading to adhesion formation. Over time, the adhesion may provide the framework for vascular ingrowth, during the process of angiogenesis[3,23,24].

The pathogenesis of adhesions involves three important trauma-induced processes (Figure 1): (1) trauma induces inhibition of the fibrinolytic and extracellular matrix (ECM) degradation systems[25,26]; (2) trauma, as well as foreign bodies, incites an inflammatory response with the production of cytokines, mainly transforming growth factor-β (TGF-β1), a key regulator of tissue fibrosis[27-29]; and (3) trauma also induces tissue hypoxia as a result of interruption of the blood supply to mesothelial cells and submesothelial fibroblasts, leading to increased expression of hypoxia inducible factor-1 α (HIF-1α)[30,31] and vascular endothelial growth factor (VEGF), responsible for collagen formation and angiogenesis[32].

MOLECULAR CROSSTALK

Sticky connected pathways

Molecular pathways involved in fibrinolysis inhibition, inflammation, and tissue hypoxia crosstalk and potentiate the effect of each. The principal molecular aberrations included in this crosstalk are the reduction of tissue plasminogen activator (tPA) and upregulation of TGF-β1 and HIF-1α.

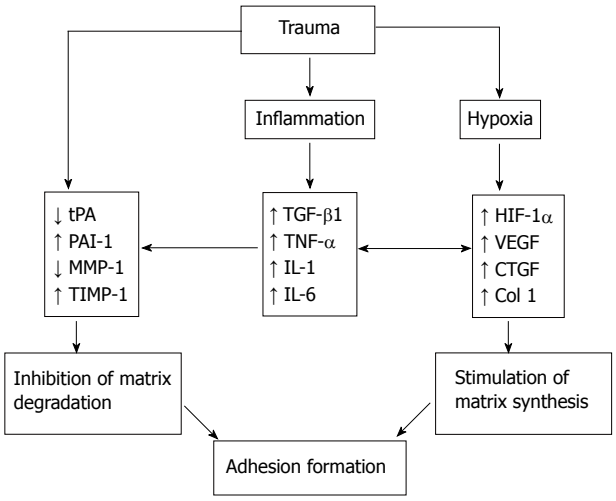

Figure 1 The role of trauma, hypoxia, and inflammation in modulating molecular crosstalk in adhesion formation. tPA: Tissue plasminogen activator; PAI-1: Plasminogen activator inhibitors 1; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitors of MMP; TGF-β1: Transforming growth factor-β; TNF-α: Tumor necrosis factor-α; IL: Interleukin; HIF-1α: Hypoxia inducible factor-1α; VEGF: Vascular endothelial growth factor; CTGF: Connective tissue growth factor.

INHIBITION OF FIBRINOLYSIS AND MATRIX DEGRADATION

The role of fibrinolysis in adhesion formation/reformation is to breakdown the fibrin clots that are formed during the healing process. The inactive proenzyme, plasminogen, is converted to plasmin by the action of tPA. Plasmin degrades fibrin and thus limits adhesion formation. Experimental and clinical studies have identified the presence of PAA in the mesothelium[33,34] and that tPA is the major (95%) physiological mediator of PAA[35,36]. Both mechanical and chemical injury reduce peritoneal PAA, with a progressive reduction in PAA in the first hours following a surgical operation, followed by complete loss of fibrinolytic activity up to 72 h after the operation[37,38]. Laparoscopic surgery also decreases peritoneal tPA[39]. This reduction in PAA is the result of reduced tPA production and increased release of plasminogen activator inhibitors 1 and 2 (PAI-1, PAI-2) by mesothelial, endothelial, and inflammatory cells[3,40]. Extensive human and animal studies confirmed the central role of altered tPA/PAI-1 balance in adhesion formation and demonstrated that this imbalance is more exaggerated in severe adhesions[25,26,34,40].

Plasmin also activates latent matrix metalloproteinases (MMPs) involved in extracellular matrix (ECM) degradation. The proteolytic activity of MMPs is regulated in part by their physiological inhibitors, tissue inhibitors of MMPs (TIMPs). It has been shown that MMPs and TIMPs are ex-

pressed in the human peritoneum, in adhesion fibroblasts, and in serosal layers of several intraperitoneal organs with and without adhesions[3,41,42]. Chegini *et al*[43] demonstrated that serosal tissue of intraperitoneal organs obtained during open surgery expresses more tissue inhibitor metalloproteinase-1 (TIMP-1) than matrix metalloproteinase-1 (MMP-1), and that adhesions express elevated levels of TIMP-1 and a lower ratio of MMP-1 to TIMP-1 compared with intact parietal peritoneum. The association between the imbalance of MMP/TIMP production and adhesion formation has been confirmed in another study in women undergoing laparoscopy[29].

INFLAMMATION AND THE ROLE OF TRANSFORMING GROWTH FACTOR-β-1

Several studies have demonstrated that during the acute phase of the inflammatory response, mesothelial cells and peritoneal macrophages produce a variety of cytokines, including TGF-β1, tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), and IL-6. These pro-inflammatory cytokines, individually and synergistically, stimulate the production of PAI-1 and reduce the synthesis of tPA by human mesothelial cells (Figure 1)[3,44-46]. TGF-β not only interacts with the fibrinolytic system and ECM, but also with many other cellular mediators involved in the process of adhesion formation. TGF-β1 overexpression by the peritoneum, as well as increased concentrations of TGF-β in the peritoneal fluid, has been associated with increased incidence of adhesion formation in both humans and animals[3,27,47,48].

Several studies demonstrated that increased TGF-β1 is associated with a reduction of tPA and an increase of PAI-1 release[27,49,50], an excess of TGF-β1 leads to an increase in the severity of adhesions formed[27,51], whereas an inhibitory antibody to TGF-β1 decreased adhesion formation[52]. TGF-β1 contributes to the synthesis of the ECM by stimulating fibroblastic cell production of collagen and fibronectin[53-55]. TGF-β also antagonizes ECM resorption by decreasing the activity of MMPs through decreasing MMP-1 and increasing TIMP-1 expression from mesothelial cells[3,56,57]. This impairment of MMP activity prevents the ECM deposition that occurs early in wound healing from being adequately remodeled and degraded when necessary, as healing progresses.

THE ROLE OF HYPOXIA IN ADHESION FORMATION

Several lines of evidence have demonstrated that peritoneal tissue hypoxia plays a key role in adhesion formation[58-61]. During laparotomy, tissue injury including trauma, desiccation, and vascular disruption (due to ligatures and other vascular hemostatic methods, including cauterization) reduces oxygen supply to the peritoneum. Laparoscopic surgery was shown to be less adhesiogenic, not only because it is less traumatic, but also due to elevated peritoneal tissue oxygen tension levels compared to those during laparotomy[58]. However, adhesions during laparoscopic surgery increase with duration of pneumoperitoneum and with insufflation pressure. These effects were attributed to desiccation and compression of the capillary flow in the superficial peritoneal layers by the pneumoperitoneum. The addition of oxygen to the insufflation gas decreases adhesion formation[59]. Moreover, supplemental perioperative oxygen was found to increase peritoneal tissue oxygen tension and to reduce the severity of adhesions[61].

Hypoxia negatively modulates all pathways involved in adhesion formation. Hypoxia decreases tPA and increases PAI expression in human peritoneal fibroblasts *in vitro*[62] and in peritoneal tissues *in vivo*[61], thereby decreasing plasmin, inhibiting lysis of fibrin, and increasing adhesion formation. The *PAI-1* gene contains oxygen responsive promoter sequences, namely hypoxia response element (HRE-1 and HRE-2), to which HIF-1α binds and induces gene expression[63]. Hypoxia was found to increase expression of TIMP-1, but not MMP-1, in both peritoneal and adhesion fibroblasts[55], thus decreasing matrix degradation. Hypoxic conditions in cultured human mesothelial cells and peritoneal fibroblasts also increased the expression of TGF-β1[55,64,65]. Hypoxia resulted in increased expression of collagen 1 mRNA in both peritoneal and adhesion fibroblasts[55,66], probably through the production of superoxide[66]. Moreover, hypoxia induces proliferation while inhibiting apoptosis in fibroblasts from adhesion, thus favoring adhesion formation[67]. Finally, hypoxia increases VEGF production through activation of HIF-1α in normal and adhesion fibroblasts *in vitro*[68] and *in vivo* in human adhesion mesothelial cells[69] and in animal adhesion tissues[30,70]. VEGF plays a central role in angiogenesis and its role in adhesion blood vessel development has been established[71]. Furthermore, TGF-β1 stimulates VEGF and connective tissue growth factor (CTGF) expression[72]. CTGF stimulates increased expression of ECM fibronectin, collagen 1, and laminin, while CTGF knockdown inhibits ECM production induced by TGF-β1 in human mesothelial cells[73].

CURRENT PREVENTION THERAPIES

Rules of disengagement

Single therapeutic strategies have failed to completely prevent peritoneal adhesions because of the multifactorial nature of adhesion pathogenesis[74]. As these multifactorial etiologies act independently and synergisctically in adhesion formation, it is imperative to simultaneously address the major molecular aberrations, including reduction of tPA and upregulation of TGF-β1 and HIF-1α, for any therapeutic strategy to be successful. The current preventive approaches of reducing surgical trauma, use of physical barriers or administration of single pharma-

Table 1 ***In vivo*** **adhesion prevention gene therapy studies**

Ref.	Vector/dose	Nucleic acid	Adhesion reduction (%)
Atta *et al*[101]	Adenovirus, 5×10^7 pfu	*tPA* gene	34
Guo *et al*[102]	Plasmid, 100 μg, sonoporation	*Smad7* gene	37
Guo *et al*[103]	Adenovirus	*Sphingosine kinase-1* gene	62
Segura *et al*[31]	Polyethylenimine cationic polymer, 2-4 nmol	siRNA HIF-1α siRNA PAI-1	36 to 52
Liu *et al*[104]	Adenovirus, 1×10^9 pfu	*HGF* gene	56

tPA: Tissue plasminogen activator; siRNA: Small interfering RNA; HIF-1α: Hypoxia inducible factor-1α; PAI-1: Plasminogen activator inhibitors 1; HGF: Hepatocyte growth factor.

cological agent or gene therapy have all failed to achieve satisfactory results.

Surgical precautions

The general surgical precautions aiming at minimizing surgical trauma include meticulous surgical techniques, delicate purposeful tissue handling, achieving optimal hemostasis, minimizing the risk of infection, and avoiding contaminants (e.g., fecal matter) and the use of foreign materials (e.g., talcum powder) when possible[74,75]. However, these surgical techniques alone are not effective.

Physical barriers

Physical barriers work by separating surgically injured tissues during the initial postoperative time period while remesothelization is occurring, a process that is usually expected to take three to five days[74]. Currently, three barriers, Interceed® (Johnson and Johnson, Gynecare, Somerville, NJ), Seprafilm® (Genzyme, Cambridge, MA), and ADEPT® (Baxter, Deerfield, IL), are approved by the Food and Drug Administration (FDA) for clinical use in the United States[74]. Although barriers have shown some success[74,76], this experience is not universally confirmed[77]. In fact, the FDA warns surgeons that when Interceed is used laparoscopically, patients have more adhesions than patients in the control group[76]. In the United States, ADEPT is only approved for laparoscopic gynecological surgery, and is contraindicated for patients with infection or allergies to cornstarch, as well as procedures involving laparotomy incision, bowel resection, or appendectomy. If used in these contraindicated procedures, patient may experience dehiscence, cutaneous fistula formation, anastomotic failure, ileus, and/or peritonitis. Thus, application and adoption of this product have been very limited[76]. Furthermore, the surgeon must predict the potential sites of adhesion formation in order to determine the placement site and to optimize barrier function[78].

Molecular therapy

A multitude of pharmacological agents, including recombinant proteins and antibodies, have demonstrated moderate success in reducing adhesion formation in different experimental adhesion models. These agents are applied locally into the peritoneal cavity, and work by correcting aberrant molecular pathways operative during adhesion development. One of the most extensively studied pharmacological agents that has demonstrated consistent success is recombinant tPA (reviewed in Ref 92)[79-92]. Experimental studies have reported reduction in adhesion formation and reformation using intraperitoneal recombinant human tPA in a variety of delivery methods and preparations, without impairing the healing of bowel anastomosis and without reduction in wound strength or causing hemorrhagic complications[79,80,92,93]. The action of tPA is localized to fibrin deposits; therefore, fibrinolytic activity is limited to this site, which prevents indiscriminate fibrinolysis[90]. Similar experiences were obtained in studies using neutralizing antibodies for PAI-1[94], TGFβ-1[45,52,95], TNF-α and IL-1[96], IL-6[97], and for VEGF[98,99]. However, these agents have short half-lives (few minutes) limiting their fibrinolytic effect for a sufficient duration of time (three to five days) until complete healing of peritoneal surfaces[92,100].

Gene therapy

Local molecular therapy is inherently limited; therefore, an alternative strategy using gene therapy has been recently employed to correct molecular aberrations induced by surgical trauma in a regulated manner during the period of remesothelialization. Postoperative peritoneal adhesion is an attractive target for gene therapy because of several inherent biological features. The disease is localized to the site of peritoneal trauma and develops over a short period of time, extending for the first few days following surgical trauma. These characteristics lend themselves perfectly to gene therapy using non-integrating vectors. The vector can be applied locally following completion of the operation, and the short duration of gene expression would cover the period of altered molecular aberrations (e.g., depressed tPA, elevated PAI-1, TGF-β1, HIF-1α, *etc.*) following surgery. Nevertheless, gene therapy for peritoneal adhesions is still in its infancy, with very few *in vivo* studies reported in the literature (Table 1).

Using different vectors, the five gene therapy studies reported in the literature were able to express therapeutic nucleic acids (transgenes or small interfering RNA) in the peritoneal tissues after intra-peritoneal administration in a rat adhesion model for at least seven days post-administration[31,101-104]. This duration of expression is enough

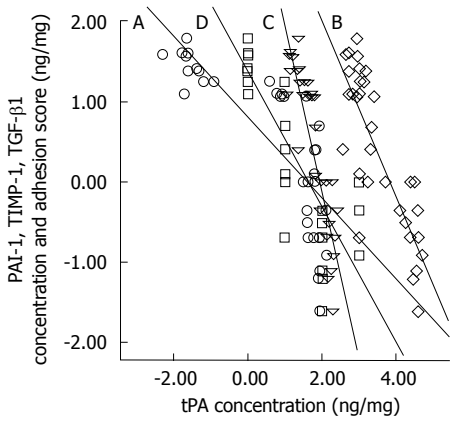

Figure 2 Correlation analysis was performed using two-tailed Spearmen rank correlation test between tPA with respect to PAI-1 (line A, marker ○), TIMP-1 (line B, marker ◇), TGF-β1 (line C, marker ▽) or adhesion score (line D, marker □). Values of tPA, PAI-1, TIMP-1, and TGF-β1 concentration in adhesion tissues were logarithmically transformed to obtain a normal distribution before statistical analysis. There was significant negative correlations ($P < 0.01$) between human tPA and either of PAI-1 (Spearman rank correlation coefficient, $r = -0.89$), TIMP-1 ($r = -0.73$), TGF-β1 ($r = -0.87$), or adhesion score ($r = -0.87$). tPA: Tissue plasminogen activator; PAI-1: Plasminogen activator inhibitors 1; TIMP: Tissue inhibitors of MMP; TGF-β1: Transforming growth factor-β.

to cover the time required for complete healing of the mesothelial cell layer of the peritoneum. The mechanism of adhesion reduction differed among these studies. Two studies showed that an adenovirus encoding the genes of hepatocyte growth factor (HGF) itself or its down-stream signaling molecule sphingosine kinase 1 (SK-1) could achieve adhesion reduction *via* a stimulatory effect on proliferation and migration of mesothelial cells[103,104]. The altered tPA/PAI-1 balance occupies a central role in adhesion formation and two studies tackled this molecular imbalance. Atta *et al*[101] used an adenovirus vector encoding human tPA, while Segura *et al*[31] employed a cationic polymer containing siRNAs to PAI-1 and HIF-1α to downregulate PAI-1, either directly or through its gene inducer, HIF-1α. The expression of TGF-β1 was attenuated by overexpressing its down-stream Smad2/3 natural inhibitor Smad7 using plasmid vector[102].

The moderate success in adhesion reduction in these gene therapy studies supports the concept that the multifactorial nature of molecular aberration during adhesion formation should be collectively and simultaneously addressed for any therapeutic strategy to be effective. This concept does not contradict, but reinforces, the established hypothesis of the central role of tPA reduction following surgical trauma in adhesion formation. In a recent study of adhesion prevention from our laboratory using an adenovirus vector encoding human tPA, we verified that overexpression of human tPA resulted in abrogation of the elevated fibrogenic molecules PAI-1, TIMP-1, and TGF-β1. The study showed that the reduction of these molecules depends on the concentration of the expressed human tPA protein[101]. Further analysis showed that there are significant negative correlations (at 0.01 level, 2-tailed) between human tPA and either of PAI-1 (Spearman's $r = -0.89$), TIMP-1 ($r = -0.73$), TGF-β1 ($r = -0.87$), or adhesion score ($r = -0.87$) (Figure 2).

Prospects

Gene therapy for the prevention of peritoneal adhesions has not been fully explored. Two potential developments for safe and effective gene therapy studies for adhesion prevention include combinatorial gene therapy and vector modifications.

COMBINATORIAL GENE THERAPY

The multifactorial nature of adhesion formation proposes that a combinatorial gene therapy would be more efficacious than a single gene therapy approach. For example, this could include overexpression of the fibrinolytic *tPA* gene together with downregulation of fibrogenic genes, such as TGF-β1 and/or HIF-1α. Overexpression is achieved through delivery of an exogenous gene, while downregulation is accomplished by the delivery of small interfering RNA (siRNA) molecules. Upon delivery, siRNAs complement with specific mRNAs resulting in their degradation, thus enabling the specific silencing of a single gene at the cellular level. As discussed above, single gene overexpression or silencing was moderately successful in reducing experimental adhesions[31,101]. The synergistic effects from the combined fibrinolysis stimulation and fibrogenesis inhibition, however, remain to be confirmed.

SAFE AND EFFICIENT VECTORS

Viral vectors

Delivery of therapeutic nucleic acid molecules to target tissues is accomplished using either viral vectors or non-viral carrier systems. Replication-deficient recombinant adenovirus vectors have become the most widely used viral vectors for *in vivo* gene transfer[101]. Adenovirus vectors have many positive attributes, including their ability to provide efficient *in vivo* gene transfer to both dividing and non-dividing cells, their high *in vivo* stability, and their non integrating nature into the host genome. These merits make adenoviral vectors suitable for proof-of-principle experimental studies. However, the clinical application of virus-mediated gene delivery *in vivo* is hampered by virus-induced acute inflammation, which could be fatal, high immunogenicity, and low tissue specificity. The broad tropism of adenovirus allows the virus to infect many cell types and is responsible for virus dissemination to distant organs. Various modifications of adenoviral vectors are underway to enhance the targeting of adenoviral vectors towards adhesion fibroblasts, which will provide effective and safe methods for localized treatment of postoperative peritoneal adhesions.

Nonviral vectors

Given the unresolved safety limitations of viral vectors, significant research efforts have been directed towards

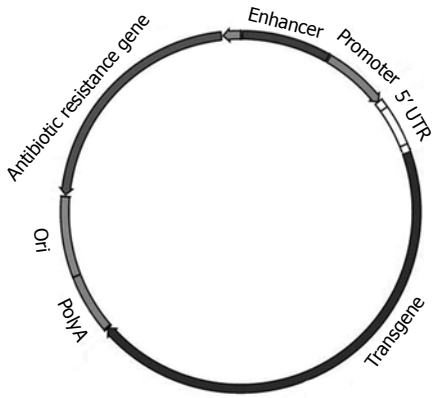

Figure 3 Skeleton of a conventional plasmid. Ori: Origin of replication.

the development of non-viral (plasmid-based) delivery systems. Plasmids are extrachromosomal genetic elements able to replicate autonomously and to be maintained in a host cell. Plasmids, the most basic forms of non-viral gene therapy, carry two main units: a eukaryotic transcription cassette and the bacterial amplification unit (Figure 3). The first bears genetic elements intended for gene expression in mammalian (eukaryotic) cells, such as the enhancer/promoter sequences for gene expression; 5' untranslated region (5' UTR), the gene of interest and polyadenylation (polyA) sequence. The bacterial amplification unit commonly contains an origin for plasmid DNA replication (ori) in bacteria and, generally, an antibiotic resistance selection marker[105]. Plasmids do not enter cells efficiently because of their large size, hydrophilic nature (due to negatively charged phosphate groups), and their susceptibility to nuclease-mediated degradation. Plasmid DNA and siRNA are stable for only 0.5 and 2 h, respectively in human serum[106]. Plasmids can be delivered to cells either naked (carrier-free) by direct injection, electroporation, ultrasound *etc.*, or complexed with cationic lipids (lipoplexes), cationic polymers (polyplexes), peptides or inorganic nanoparticles[107]. Two promising recent modifications of plasmid vectors, minicircles and CpG-depleted vectors, are briefly discussed below. Evaluating the rapid progress in the field of cationic liposomes and polymers is, however, beyond the scope of this brief review, and the reader is referred to excellent recent reviews[107,108].

Minicircle vectors

Limitations of conventional DNA plasmid vectors are related to their size (> 3 kb). Moreover, bacterial sequences contain immunotoxic cytidine-phosphate-guanosine (CpG) dinucleotides motifs, which are approximately four times more prevalent in bacterial than mammalian DNA. Bacterial CpG dinucleotides have been identified to be major contributors to the low and short-lived transgene expression (transgene silencing) in vertebrates after non-viral gene delivery. These bacterial sequences can also interfere with short hairpin RNA (shRNA, precursor of siRNA) expression[106,109]. To overcome these limitations, highly safe and efficient vector systems for gene transfer in eukaryotic cells called minivectors (minicircles) were developed[110]. Minicircles are supercoiled minimal expression cassettes, derived from conventional plasmid DNA by site-specific recombination *in vivo* in Escherichia coli. As a result, two well-defined circular molecules are generated from the parent conventional plasmid, termed minicircle (mammalian expression cassette) and miniplasmid (bacterial backbone elements). Further purification of the minicircle renders it therapeutically applicable[105]. Thus, minicircle DNA lacks the bacterial backbone sequence consisting of an antibiotic resistance gene and an origin of replication. Minicircle DNA is low in immunogenicity due to its lower content of bacterial unmethylated CpG dinucleotides. In addition to their improved safety profile, minicircles have been shown to greatly increase the efficiency of transgene expression in various *in vitro* and *in vivo* studies, compared to the conventional plasmid with the same transgene expression cassette. It has been reported that a minivector incorporating short hairpin RNA efficiently transfected adhesion fibroblasts and was shown to be stable in human serum for > 48 h[107].

CpG-depleted vectors

Bacterial DNA is rich in unmethylated CpG dinucleotides, in contrast to mammalian DNA, which contains a low frequency of CpG dinucleotide, which are mostly methylated. Recognition of unmethylated CpGs present in the bacterial backbone could trigger an innate immune response following detection in the endosome by toll-like receptor 9 (TLR9)[109] and initiate a signaling cascade, leading to the production of proinflammatory cytokines. As plasmids used in *in vivo* gene therapy studies are produced in *Escherichia coli* (*E. coli*), their CpGs are unmethylated and induce immune responses through this host defense mechanism. Recently, plasmids that are completely devoid of CpG dinucleotides have been developed. These plasmids yield high levels of transgene expression both *in vitro* and *in vivo*, and, in contrast to CMV-based plasmids, allow sustained expression *in vivo*[111,112]. In these CpG-free plasmids, all elements required for replication and selection of the plasmid in *E. coli* and for gene expression in mammalian cells (e.g., promoter, polyadenylation signal, reporter gene, *etc.*) either naturally lack CpG dinucleotides, were modified to remove all CpGs, or are entirely synthesized.

CONCLUSION

Gene therapy for the prevention of postoperative peritoneal adhesions is still in its infancy. The potential applications of this strategy have not been fully explored. The recent explosive progress in advanced nonviral gene delivery systems, coupled with the newly developed less immunogenic and more efficient expression plasmids, will undoubtedly accelerate research studies in gene therapy for peritoneal adhesions.

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REVIEW

Role of (^{18}F) 2-fluoro-2-deoxyglucose positron emission tomography in upper gastrointestinal malignancies

Elizabeth C Smyth, Manish A Shah

Elizabeth C Smyth, Manish A Shah, Department of Gastrointestinal Oncology, Weill Cornell Medical College, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, United States
Manish A Shah, Department of Gastrointestinal Oncology, Weill Cornell Medical College, New York-Presbyterian Hospital, New York, NY 10065, United States
Author contributions: Smyth EC and Shah MA contributed equally to this paper.
Correspondence to: Manish A Shah, MD, Director, Department of Gastrointestinal Oncology, Weill Cornell Medical College, New York-Presbyterian Hospital, 1305 York Avenue, New York, NY 10065, United States. manish.a.shah.md@gmail.com
Telephone: +1-646-9622547 Fax: +1-646-9621607
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Abstract

The role of whole-body FDG [(^{18}F) 2-fluoro-2-deoxyglucose] positron emission tomography (PET) scanning as an imaging modality in the management of patients with malignancy has evolved enormously over the past two decades. FDG-PET has demonstrated significant efficacy in the staging, prognostication and detection of occult metastatic disease in malignancies of the gastrointestinal tract, in addition to assessment of the response to cytotoxic chemotherapy in a more timely manner than has traditionally been possible by more conventional imaging tools. The sensitivity and specificity of FDG-PET for the detection and staging of malignancy depend not only on the site and size of the primary tumor and metastases, but also on histological cell type, reflecting underlying disparities in glucose metabolism. The metabolic response to neo-adjuvant chemotherapy or to chemo-radiotherapy in cancers of the gastro-esophageal junction or stomach has been demonstrated in several prospective studies to correlate significantly with both the histological tumor response to treatment and with consequent improvements in overall survival. This may offer a future paradigm of personalized treatment based on the PET response to chemotherapy. FDG-PET has been less successful in efforts to screen for and detect recurrent upper gastrointestinal malignancies, and in the detection of low volume metastatic peritoneal disease. Efforts to improve the accuracy of PET include the use of novel radiotracers such as (^{18}F) FLT (3-deoxy-3-fluorothymidine) or ^{11}C-choline, or fusion PET-CT with concurrent high-resolution computed tomography. This review focuses on the role of FDG-PET scanning in staging and response assessment in malignancies of the upper gastrointestinal tract, specifically gastric, esophageal and pancreas carcinoma.

Key words: Positron emission tomography; Gastric cancer; Esophageal cancer; Pancreas cancer

Peer reviewer: Dr. Cem Onal, Department of Radiation Oncology, Baskent University Faculty of Medicine, Adana 01120, Turkey

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INTRODUCTION

Whole-body positron emission tomography (PET) scanning after the administration of (^{18}F) 2-fluoro-2-deoxyglucose (FDG) has emerged as a promising new imaging modality in the management of patients with malignancy. The role of FDG-PET scanning in upper gastrointestinal (GI) malignancies has evolved tremendously over the past two decades. Like most imaging modalities, FDG-PET initially made its mark in staging for preoperative risk assessment, prognostication, and in evaluation of

distant metastatic disease. FDG-PET scanning has also improved our ability to identify occult metastatic disease in a number of malignancies, including malignancies of the upper GI tract. When considering glucose uptake as a surrogate for metabolic activity, another important application of FDG-PET scanning is therapeutic response assessment. Traditional computed tomography (CT) scanning has been the mainstay for assessment of the effectiveness of cytotoxic therapy in solid tumor oncology; however with the advent of FDG-PET, it has been increasingly apparent that this new modality may also provide an assessment of the therapeutic effectiveness of cytotoxic therapy, and possibly at an earlier time point.

This review focuses on the role of FDG-PET scanning in staging and therapeutic response assessment in malignancies of the upper GI tract, specifically gastric and esophageal carcinoma.

SCIENCE OF FDG-PET AND CHANGES IN FDG UPTAKE

FDG uptake is considered as a surrogate for the metabolic activity of a malignancy, specifically linked to glucose metabolism in malignant cells[1]. The role of FDG-PET imaging, in fact, may be related to the Warburg effect-the observation made by Otto Warburg in 1924 that suggested that cancer cells metabolize glucose differently from normal non-malignant cells[2]. Specifically, cancer cells tend to grow and metabolize nutrients independent of growth factor stimulus, but not in the most efficient manner for ATP generation, but rather in a manner that would support the acquisition of building blocks for continued, uncontrolled cell division and growth[2]. Central to this hypothesis is dysfunction of the phosphoinositide 3-kinase signaling pathway, commonly identified as pathologic in a majority of malignancies, and which is central to both growth control and glucose metabolism. A change in glucose metabolism, as identified by FDG-PET serial imaging, may therefore uniquely predict subsequent cell death[1].

Glucose uptake by malignant cells is largely mediated by the GLUT-1 transporter[3]. In a study of 60 patients with squamous cell carcinoma of the esophagus, Hiyoshi *et al*[4] demonstrated that GLUT-1 expression was correlated with the depth of tumor, lymph node metastasis and pathological stage, in addition to FDG avidity on PET imaging. Mu *et al*[5] correlated the standardized uptake value (SUV) with the expression of GLUT-1 and the Ki-67 proliferative marker, and found that with increasing clinical stage and pathological dedifferentiation, the expression of both markers increased concurrently, indicating an association with tumor aggressiveness. Tohma *et al*[6] demonstrated that FDG uptake may have a more significant association with the intracellular enzyme hexokinase-2 expression than with GLUT-1 expression. In contrast, FDG uptake is not associated with cyclin D1, p53, epidermal growth factor receptor or vascular endothelial growth factor expression in esophageal tumors[7].

ESOPHAGEAL CARCINOMA

Role of PET in staging the depth of disease-esophageal carcinoma

Clinical significance of T stage: Penetration of the primary tumor through successive layers of the walls of the esophagus is described using the T stage of the tumor. Deeper levels of mucosal involvement are associated with a higher risk of nodal and distant metastasis, and diminishing overall survival. The location of the primary tumor within the esophagus has particular relevance to the draining lymph node stations for that area. Nodal metastasis beyond the locoregional nodes may render the patient unresectable as a result. Early cancers (T2 or less) may undergo primary surgical resection. Those tumors with T3 or greater depth of penetration may undergo preoperative chemotherapy or chemoradiotherapy with a view to future resection, or definitive combined modality therapy.

FDG-PET and T stage

In an initial study of FDG-PET in the assessment of esophageal cancer by Flamen *et al*[8], FDG-PET detected 70 out of 74 esophageal lesions. It failed to detect 4 small (< 8 mm) T1 lesions. This study demonstrated no correlation between the SUV and the T stage. A retrospective series from Japan similarly demonstrated superior sensitivity of PET for the detection of T2 or greater disease; 25/25 patients with T2 or greater tumors had FDG uptake, compared to 0/7 with T1 tumors. Significant correlations with increased SUV uptake were seen with both the size of the primary and with the depth of tumor invasion[9].

In a prospective series of 81 patients who underwent surgery with no preoperative treatment, PET detected the primary lesion in 43% of pT1 tumors. Sensitivity was significantly better for pT1b disease at 61%, compared with 18% for pT1a. PET positivity increased with increasing levels of tumor invasion, being 83% at T2, 97% at T3 and 100% at T4[10]. Importantly, in another study examining patients with early stage tumors who underwent primary surgical treatment, PET-CT could not distinguish between those with carcinoma *in situ* (Tis) *vs* those with T1 disease, with FDG uptake in 5/11 (45%) and 26/47 (55%) respectively. The investigators noted a trend towards both increased frequency of FDG uptake and increased SUV with increasing depth of invasion.

It may be concluded from this data that PET, and indeed PET-CT, is an inadequate modality for assessing depth of tumor penetration within the mucosal wall of the esophagus, and also that it cannot distinguish adequately between carcinoma *in situ* and invasive disease. However, with increasing depth of invasion, an FDG-PET scan is increasingly likely to identify the malignancy.

In addition, FDG avidity on FDG-PET scans should be taken in context due to the small but real rate of false positive scans. Specifically, areas of increased FDG uptake within the esophagus may have an alternate cause such as chemotherapy or radiation-induced esophagitis, candida or other benign causes[11-14]. PET lacks the specificity to differentiate between these conditions, under-

Table 1 Prospective studies comparing the accuracy of positron emission tomography with computed tomography and/or endo-ultrasonography for the detection of lymph-node metastases

Ref.	Yr	Histology	*n*	Imaging	Sensitivity (%)	Specificity (%)
Flamen *et al*[114]	2000	SCC/AC	74	PET	39	97
				CT	63	88
				EUS	22	96
				EUS/CT	54	90
Lerut *et al*[115]	2000	SCC/AC	42	PET	22	91
				CT/EUS	83	45
Yoon *et al*[116]	2003	SCC	81	PET	30	90
				CT	11	95
Sihvo *et al*[18]	2004	AC	55	PET	35	91
				CT	42	45
				EUS	85	60
Lowe *et al*[19]	2005	SCC/AC	75	PET	82	60
				CT	84	67
				EUS	86	67
Shimizu *et al*[20]	2009	SCC	20	PET-CT	11-50	85-100
				Thin slice CT	22-100	69-100

PET: Positron emission tomography; CT: Computed tomography; EUS: Endoscopic ultrasound; SCC: Squamous cell carcinoma; AC: Adenocarcinoma.

scoring the inadequacy of this approach. Due to these factors, endoscopic ultrasound (EUS) is the preferred method for assessment of the depth of invasion of the primary tumor through the wall of the esophagus. This has been demonstrated in a meta-analysis of 49 studies to have a sensitivity of 81%-90% for T staging and a specificity of 99%[15]. EUS is limited by inability to pass through stenotic tumors in these cases, PET or PET-CT based imaging may serve as a useful adjunct.

Role of PET in staging nodal disease-esophageal cancer

Clinical significance of nodal stage: Nodal status in esophageal cancer is determined by the presence or absence of involved locoregional lymph nodes. The regional designation of a lymph node relates to its anatomical relationship to the primary tumor. Tumors of the upper third of the esophagus drain to superior mediastinal and cervical lymph nodes. Tumors of the middle third drain both superiorly and inferiorly to paratracheal, hilar, subcarinal, periesophageal, and pericardial lymph node stations. Tumors of the lower third of the esophagus drain to lymph node basins in the lower mediastinum and celiac areas Patients with non-regional lymph node spread have a worse prognosis than those with locoregional spread only, but better than those with distant metastases.

Initial reports of PET showed promise due to apparent increased sensitivity in the detection of lymph node metastasis when compared to CT[16]. However this may have been due to the use of outdated CT technology and techniques, and this initial promise with respect to increased sensitivity has not been sustained in well designed prospective studies.

In an initial report, Flamen *et al*[8] reported that 74 patients demonstrated a lower sensitivity of PET for the detection of regional lymph node metastasis when compared to EUS (81% *vs* 33%) but with a non-significant trend towards higher specificity (84% *vs* 69%). PET showed a higher specificity than CT and EUS combined when staging both regional and non-regional lymph node metastases for esophageal cancer. In a prospective study of 58 patients comparing CT and PET in the detection of lymph node metastasis within the abdomen by Kneist *et al*[17], the investigators observed a sensitivity of only 24% for PET compared to 73% for CT. Sensitivity of PET was significantly less in the area of the lesser curvature and the celiac trunk. Specificity was 75% and 95%, respectively. Within the thorax, PET demonstrated an improved but still inferior sensitivity (42% *vs* 75%) and again a superior specificity to CT. A prospective evaluation of CT, EUS and PET by Sihvo *et al*[18] demonstrated that EUS had a higher sensitivity for the detection of nodal disease (85%) than CT or PET (42% and 35%). The combination of CT, EUS and PET did not appreciably increase the sensitivity of the assessment. Neither was there any synergy between modalities with respect to specificity. A 2005 study performed by Lowe *et al*[19] comparing CT, PET and EUS for the staging of esophageal cancer showed comparable sensitivities between the three modalities for the detection of nodal disease (82%-86%). Specificity was also not significantly different at 67% for CT and EUS, and 60% for PET.

Progress in the development of both CT and PET imaging may lead to improvements in the diagnostic accuracy of both modalities. A recent study comparing thin slice CT to PET-CT in the detection of subclinical lymph node metastasis in patients with operable squamous cell carcinoma demonstrated the superiority of CT for the detection of disease at all lymph node stations, with the caveat that sensitivity appeared to decrease from the cervical area (100%) to the abdominal area (22%). Specificity was high for both CT and PET in the cervical and abdominal lymph node basins, with superior specificity for PET demonstrated only within the mediastinum[20].

The results of the above studies are described in Table 1. In order to better characterize these heterogenous results, a meta-analysis was performed by van Westreenan *et al*[50]. This included both prospectively and retrospectively obtained data. Pooled sensitivity for the detection of locoregional lymph node metastases was 51% (range, 8%-92%) with pooled specificity of 84% (range, 67%-100%)[21]. The low sensitivity of PET in prospective studies may be due to a selection bias in many cases. These results may be biased by the inclusion only of apparently early stage patients who proceeded immediately to surgery. Those who required preoperative chemotherapy and/or radiation were excluded, leading to an over-representation of solely micrometastatic foci, which are less reliably detected. For reasons of this relatively low sensitivity of PET for locoregional disease, and due to its excellent specificity, FDG-PET is better as an adjunct to conventional

Table 2 Prospective studies comparing the accuracy of positron emission tomography with computed tomography and/or endo-ultrasonography in the detection of distant metastases

Ref.	Yr	Histology	*n*	Imaging	Sensitivity (%)	Specificity (%)
Flamen *et al*[8]	2000	SCC/AC	74	PET	71	90
				CT	41	83
				EUS	42	94
				EUS/CT	47	78
Lerut *et al*[115]	2000	SCC/AC	42	PET	77	90
				CT/EUS	46	69
Sihvo *et al*[18]	2004	SCC	81	PET	35	91
				CT	42	45
Heeren *et al*[25]	2004	SC/AC	74	PET	71	98
				CT	21	98
				CT/EUS	29	96
Lowe *et al*[19]	2005	SCC/AC	75	PET	81	91
				CT	81	82
				EUS	73	76

PET: Positron emission tomography; CT: Computed tomography; EUS: Endoscopic ultrasound; SCC: Squamous cell carcinoma; AC: Adenocarcinoma.

imaging modalities for the detection of lymph node metastases rather than a comprehensive staging investigation in its own right.

Efforts to improve accuracy of PET in the detection of lymph node metastasis

The limited spatial resolution of PET may lead to difficulties due to the fact that uptake within lymph nodes close to the primary tumor may be difficult to distinguish from the tumor itself. Fusion PET-CT and correlation with metabolic and tumor-related parameters may offer superior sensitivity for the detection of nodal disease. A 2009 study by Roedl *et al*[22] compared fusion PET-CT with PET viewed side by side with CT images, in addition to axial tumor area, tumor width diameter and SUV uptake. Fusion PET-CT was more sensitive and more specific for the detection of lymph node metastasis at 70% *vs* 62% and 95% *vs* 91%, respectively. Sensitivity and specificity of 87% and 85% were increased by the addition of tumor diameter measurements. However when qualitative visual analysis was added to quantitative tumor dimension measurement in addition to PET-CT the sensitivity was 96% and the specificity 95%.

Dual time PET may assist in the differentiation between benign and malignant lesions, and may also improve the accuracy of detection of lymph node metastasis in esophageal cancer. Small malignant lesions and malignant lymph nodes show an increase in SUV uptake over time, whereas benign disease does not, and shows an early peak only. An improvement in diagnostic accuracy from 83% to 91% was seen with dual time imaging of squamous cell carcinomas of the thoracic esophagus. In addition, false positive uptake in the lung hilum due to inflammatory processes was distinguished from malignant disease in 19/42 (45%) of patients using this method[23].

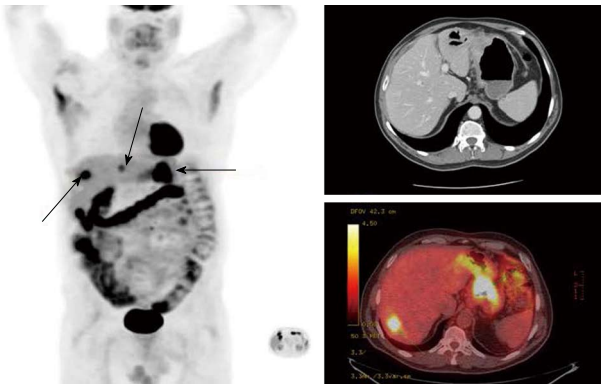

Figure 1 (^{18}F) 2-fluoro-2-deoxyglucose-positron emission tomography/computed tomography image of a patient with a proximal gastric cancer and occult liver metastasis. The liver lesion was not identified on the corresponding staging computed tomography.

DETECTION OF METASTATIC ESOPHAGEAL CANCER USING FDG-PET

PET finds a niche in the detection of metastatic disease, where its performance is superior than in the detection of the depth of the primary lesion or of locoregional lymph node involvement of esophageal carcinoma (Table 2).

An initial prospective study by Luketich *et al*[24] demonstrated a sensitivity of PET for detection of metastatic disease of 69% with a specificity of 93.4% and an overall accuracy of 84%. Following this, Flamen *et al*[8] demonstrated that FDG-PET had a superior accuracy for the detection of metastatic disease compared to combined CT and EUS (82% *vs* 64%), largely driven by the higher sensitivity of PET (74% *vs* 47%). PET correctly upstaged 15% of patients from M0 to M1 disease. The study by Lowe *et al*[19] demonstrated similar sensitivity of PET and CT at 81%, and superior specificity for PET. This may relate to improvements in CT scanning techniques in recent years.

A 2004 study by Heeren *et al*[25] demonstrated that PET upstaged up to 20% of patient to M1 disease. The accuracy of CT was 86% compared to CT/EUS at 69%. All three modalities combined provided an accuracy of 92%. In this study 13% of patients in whom M1 disease was detected on PET were spared an unnecessary surgical procedure, however 87% did require laparoscopy to confirm PET positive findings underscoring the importance of cytological confirmation of metastatic disease. In a combined analysis of 452 patients from 11 studies the pooled sensitivity and specificity for the detection of metastatic disease by PET was 67% (95% confidence interval (CI): 58%-76%) and 97% (90%-100%) respectively. Figures 1 and 2 demonstrate the detection of occult liver (Figure 1) and bone (Figure 2) metastases by FDG-PET/CT not seen on conventional CT imaging.

IS PET PREDICTIVE OF SURVIVAL IN ESOPHAGEAL CANCER?

Many studies have examined the relationship between

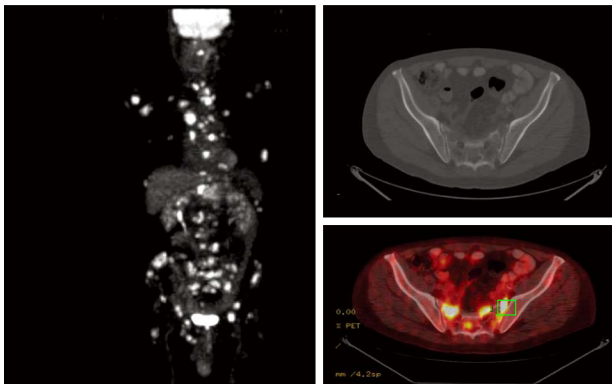

Figure 2 (^{18}F) 2-fluoro-2-deoxyglucose-positron emission tomography/computed tomography detects diffuse bony metastases not seen on staging computed tomography.

SUVmax and survival. In a recent systematic review, all 12 studies selected for inclusion demonstrated that a higher SUVmax of the primary tumor was associated with inferior survival, however only seven of these reached statistical significance. In a meta-analysis of disease-free and overall survival, the hazard ratios for disease recurrence and death were 2.52 and 1.86, respectively, for those with a higher than median SUV[26]. This correlation with peak SUV and survival may hold true even for those with apparently early stage disease[27].

SUVmax is also often significantly correlated with pathological stage, acting as a potential confounder. On multivariate analysis in several smaller studies, peak SUV was significantly associated with survival in univariate but not multivariate analysis, and thus did not emerge as an independent risk factor[28,29]. However in a large retrospective study of 184 patients with operable esophageal cancer, where SUVmax was significantly correlated with the stage of the primary tumor, lymph node status, and presence of metastasis in univariate analysis, on multivariate analysis SUV remained independently and significantly associated with overall survival when correcting for pathological stage of disease. The 5-year overall survival for those with an SUVmax $\geqslant$ 4.5 was 47% compared to 76% in those with an SUV $\leqslant$ 4.5[30]. It should be noted that the majority (91%) of patients in this study had a diagnosis of squamous cell carcinoma, and that these results contrast sharply with those published by Rizk *et al*[31] in a retrospective series of 189 patients with adenocarcinoma of the distal esophagus or gastro-esophageal (GE) junction who underwent chemoradiation as a primary treatment, in which they failed to show any association between survival for those with a high or a low SUVmax. Those with a high SUVmax did however show a superior response to chemoradiation This led the authors to conclude that although high SUVmax was correlated with inferior survival following resection in their earlier study, because high baseline SUVmax was also associated with a superior response to chemoradiation, this acted as an equalizing factor with respect to survival.

Altogether, these data suggest that high SUVmax is most likely to be associated with increased tumor stage and size of lesion. Whether SUVmax is an independent predictor of patient outcome (specifically independent of tumor stage) is not sufficiently validated.

ROLE OF FDG-PET IN RADIOTHERAPY TREATMENT PLANNING FOR ESOPHAGEAL CANCER

The gross tumor volume (GTV) must be accurately delineated in order to successfully treat the area of malignancy. However, conventional CT scanning has a low discriminatory value for this purpose. FDG-PET has been investigated in order to assess whether this improves the accuracy of this delineation. Excellent correlation has been demonstrated between preoperative FDG-PET and EUS measurements of tumor length and measurements of the same resected surgical specimen[32]. The addition of FDG-PET to conventional CT planning may lead to increases or reductions in the GTV of up to 20%, and changes in the planning target volume in over half of patients[33,34]. Modifications of GTV are most often seen in the longitudinal direction[35], however this may also change based on detection of suspicious lymphadenopathy outside the original planned treatment field[36]. Improved accuracy in GTV delineation may lead to changes in radiation dose intensity to critical structures such as the heart and lungs[33,37], whereas utilization of CT alone may lead to undertreatment of FDG-PET avid disease[34]. However, due to a lack of standardization of FDG-PET assessments of GTV and the presence of significant interobserver variation, the use of FDG-PET is not routine in radiotherapy treatment planning, nor has this been validated in terms of improved outcomes such as survival or locoregional tumor control. A prospective trial is ongoing in this regard (NCT01156831)[38].

DOES SUV PREDICT RESPONSE TO CHEMORADIOTHERAPY?

Several studies have examined whether the change in SUV of the primary tumor with chemotherapy or chemoradiotherapy is useful in determining the response to the intervening therapy. A large proportion of studies have been prospective, but were limited in their scope of analysis to some extent by small numbers. Each study evaluated a different treatment regimen. Most studies used pathological response as the gold standard for evaluation of chemotherapy efficacy. This is commonly measured using the Mandard system[39] or a simple modification of this system, where pathological response is classified according to the percentage of viable tumor cells remaining, with non-responders having > 10% tumor cells remaining, partial response 0%-10%, and complete responders 0% viable tumor cells.

A first prospective study in 2001 by Weber *et al*[40] of

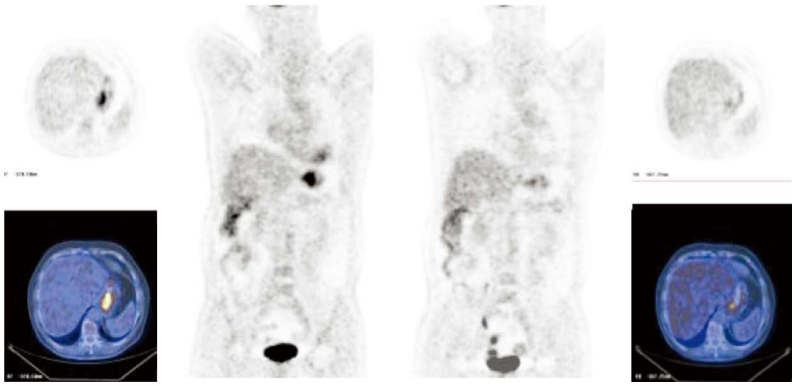

Figure 3 (^{18}F) 2-fluoro-2-deoxyglucose-positron emission tomography response in a patient with a proximal gastric cancer receiving chemotherapy.

40 patients with adenocarcinoma of the GE junction and gastric cardia demonstrated a median reduction in SUV of responders of more than three times that of non-responders and was significantly correlated with pathological response ($P < 0.001$). Response was also significantly associated with survival. Those with no response had a 2-year survival of 37% *vs* 60% in responders. Figure 3 demonstrates a sample FDG-PET/CT response for a patient with a proximal gastric adenocarcinoma.

A prospective trial by Ott *et al*[41] used a predetermined level of reduction in SUV to determine the cut-off point for metabolic responder *vs* non-responder. This had been previously determined to be a reduction of 35% from baseline, which had been demonstrated to have a sensitivity and specificity of 93% and 95%, respectively, for the detection of a pathological response[40]. Sixty five patients with locally advanced GE junction tumors undergoing preoperative chemotherapy were enrolled. Baseline tumor FDG uptake was 8.1 ± 3.4 SUV for assessable patients. SUV uptake significantly decreased to 5.4 ± 2.0 (approximately 33%) in the follow-up scan. Eighteen patients were classified as metabolic responders and 38 as metabolic nonresponders. The pathological response was highly significantly correlated with the metabolic response ($P < 0.001$); 44% of patients with a metabolic response had a pathological response, compared to 5% of metabolic non-responders. Median overall survival for non-responders was 18 mo, significantly shorter than overall survival for the group as a whole at 32 mo. Median survival for metabolic responders had not yet been reached at the time of publication.

A similar study was performed at Memorial Sloan Kettering Cancer Center as a validation study, and reported in abstract form in 2007[42]. In this study, patients with locally advanced but resectable gastric/GE junction adenocarcinoma received preoperative chemotherapy with irinotecan and cisplatin for two cycles. An FDG-PET CT scan was performed at baseline and again at day 15 and day 35. This study confirmed the results initially reported by Weber *et al*, demonstrating that a significant drop in SUV from baseline was associated with the pathologic response to therapy as well as with patient survival[42].

The primary utility of a change in FDG-PET SUV from baseline as a marker for response to chemotherapy and subsequently survival is that this information is available early in the treatment plan, and thus could potentially be used in order to guide future management. This approach was taken by Lordick *et al*[43] in the MUNICON trial. This study recruited 119 patients with locally advanced tumors of the GE junction undergoing preoperative chemotherapy. Patients who did not meet a predefined metabolic response level on FDG-PET of a 35% reduction from baseline SUVmax 2 wk after commencing treatment did not continue with chemotherapy but proceeded directly to surgery. Metabolic responders completed the course of preoperative chemotherapy and then proceeded to surgery; 49% of patients were metabolic responders and 51% were metabolic non-responders. Of the metabolic responders, 58% achieved a major histological response, with 0% in the non-responders. R0 surgical resection was possible in 96% of metabolic responders and in 74% of metabolic non-responders. On pathologic assessment, metabolic responders demonstrated earlier stage tumors than metabolic non-responders. Metabolic non-responders had a median event-free survival of 14.1 mo compared to 29.7 mo in metabolic responders. It was noted that metabolic responders who did not have a pathological response had survival comparable to those who were metabolic non-responders, implying that a metabolic response was necessary but not sufficient for improved survival[43].

In a cross trial comparison between the original study by Ott *et al*, where chemotherapy was continued despite a metabolic non-response, and MUNICON where non-responders proceeded directly to surgery, amongst those patients that went on to complete surgical resection, survival between non-responders in both groups was similar. This suggests that, amongst metabolic non-responding patients, patient survival was unaffected (either adversely or positively) by continuing with ineffective chemotherapy or by stopping ineffective chemotherapy and proceeding early to surgery. These results have led to an ongoing clinical trial in which failure to respond to initial induction chemotherapy with a reduction in SUV on

PET is followed by introduction of a salvage regimen of non-cross resistant chemotherapy in an effort to improve outcome (NCT00737438 on clinicaltrials.gov; Memorial Sloan Kettering study, IRB 08-081).

In contrast, in a study of 32 patients with esophageal/GE junction adenocarcinoma, a FDG-PET scan performed following a week of chemoradiation failed to detect any significant difference between pathologic responders and non-responders with respect to changes of SUVmax on PET[44]. This may in fact be due to the timing of the PET as radiation is known to have a "stunning" effect with respect to FDG uptake, irrespective of further cell kill, which may cause bias in an interpretation performed at an early interval following radiation.

These studies suggest that the utility of FDG-PET in response assessment in esophageal/GE junction adenocarcinoma remains to be verified at this time, but that it is a potentially promising modality to begin "individualized" care for patients with upper GI malignancies (namely esophageal and gastric adenocarcinoma). It should be noted that the response of PET to chemotherapy when compared with that of CT may lead to clinical confusion, such as when a lesion improves by PET criteria, but fails to shrink or may even enlarge slightly by traditional RESIST criteria[45]. Recently proposed guidelines for response assessment in solid tumors suggest that PET progression may be defined as an SUV increase of $\geqslant$ 20% in a region 1 cm or larger in diameter, whereas a response be defined as a decline in SUV of $\geqslant$ 30% in such a region[46]. Such a guideline would seem to be a good starting point for evaluation of the PET response in many solid tumor malignancies, but will need prospective validation.

FDG-PET FOR THE DETECTION OF ESOPHAGEAL CANCER RECURRENCE

The accuracy of CT and magnetic resonance imaging (MRI) for the detection of recurrent disease, particularly within the area of the initial primary tumor may be decreased by post-surgical or post-chemoradiation related changes such as fibrosis, edema, and inflammation. Guo *et al*[47] followed 112 patients with resected squamous cell carcinoma of the esophagus for recurrence with FDG-PET/CT. PET demonstrated excellent sensitivity at local, regional and distant sites of metastases (96.9%, 85.9% and 90.5%, respectively), but lower specificity for local-regional recurrence (50%, 92.2% and 89.9%, respectively). Of note, five out of nine false positive FDG-PET scans were identified in the area of the surgical anastomosis. A French study examined the routine use of FDG-PET in the prospective follow-up of resected esophageal cancer patients[48]. This study demonstrated that for the detection of locoregional recurrence, PET had a higher sensitivity, slightly lower specificity and a superior accuracy than CT (100% *vs* 65%, 85% *vs* 91%% and 91% *vs* 81%, respectively). PET was also superior to CT in the detection of local metastasis. No patient had a negative PET and a recurrence detected by another modality, i.e., there were no false negative PET scans in this study, leading to a 100% negative predictive value. As this recently published study is the first examining the prospective use of PET to detect recurrence in asymptomatic patient, it is too early to comment on whether changes in management based on this strategy will lead to improvements in patient outcomes.

COMPARISON OF FDG-PET AND OTHER PET TRACERS IN THE DIAGNOSIS AND MANAGEMENT OF ESOPHAGEAL CANCER

FDG is not a tumor specific radiotracer, and this leads to the drawback of false positive uptake in areas of inflammation or infection by neutrophils and macrophages, i.e., when there is contamination of the malignancy with other actively dividing or metabolically active cells. An alternative to FDG-PET is (^{18}F) FLT (3-deoxy-3-fluorothymidine) which is trapped intracellularly following phosphorylation by thymidine kinase 1 into (^{18}F) FLT-monophosphate, forming the rationale for the use of FLT as a proliferation tracer[49]. A study by Westreenan *et al*[50] compared the efficacy of FLT *vs* FDG in the detection of esophageal cancer and demonstrated increased uptake for FDG rather than FLT (FLT-PET missed 20% of primary esophageal tumors in this study). FDG-PET also detected a synchronous primary rectal tumor in one patient, which was not detected by FLT-PET. In addition, there was no correlation between uptake of FLT and Ki-67, a marker of proliferation. For this reason, FDG remains the preferred radiotracer for use in the diagnosis and management of patients with esophageal cancer[50].

^{11}C-choline is a small molecule that is integrated into the cell membrane as phosphatidylcholine and serves as a marker of cell membrane metabolism. Because of late urinary excretion, it has been examined in genitourinary tumors such as prostate cancer[51]. ^{11}C-choline has been investigated in two studies of esophageal cancer. Kobori *et al*[52] studied squamous cell carcinoma of the upper esophagus and claimed a superior sensitivity for choline-PET in the detection of primary tumors and nodal metastases in the mediastinum (94% and 88%, respectively). Specificity was not reported. In this study the sensitivity of FDG-PET was 34% and 38% for the primary tumor and nodal involvement, which is somewhat lower than the literature median. These results contrast with those of Jager *et al*[53], who studied a more diverse group of esophageal and GE junction adenocarcinomas in addition to squamous cell carcinoma of the esophagus and GI stromal tumors. They demonstrated the superiority of FDG-PET, with a sensitivity of 100%, 67%, and 100% for the detection of primary tumor, locoregional and lymph node metastases, respectively, compared to 73%, 60%, and 75%, respectively, for choline-PET. Imaging in the abdominal area

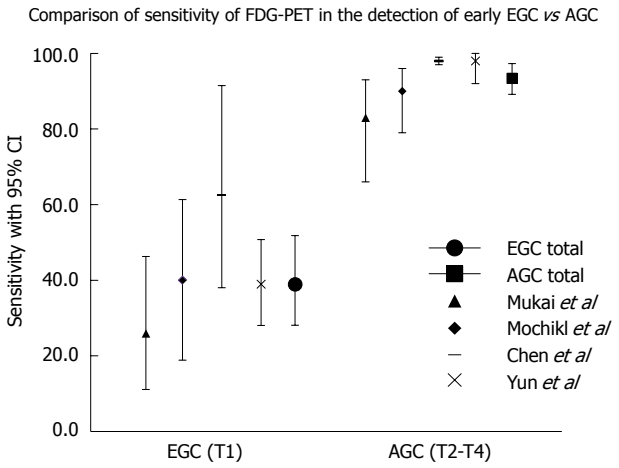

Figure 4 Sensitivity of (^{18}F) 2-fluoro-2-deoxyglucose-positron emission tomography to identify primary early and advanced gastric carcinoma. FDG-PET: (^{18}F) 2-fluoro-2-deoxyglucose-positron emission tomography; EGC: Early gastric cancer; AGC: Advanced gastric cancer; CI: Confidence interval.

using choline-PET is limited by the high background uptake of this agent by the liver.

GASTRIC ADENOCARCINOMA

Gastric cancer remains the most common GI malignancy worldwide, responsible for approximately 934 000 new diagnoses annually (8.6% of new cancer cases) and 700 349 deaths worldwide annually[54]. Gastric cancer may be distinguished anatomically such that proximal tumors (associated with chronic reflux and obesity) have worse prognosis than distal tumors which are more commonly associated with chronic infection by *Helicobacter pylori*[55]. Alternatively, gastric cancer may also be distinguished histopathologically as diffuse, intestinal, or mixed histology which describes the pattern of spread of the primary tumor[56]. Based on these distinctions, an emerging concept in understanding the biology and physiology of gastric cancer is that it likely reflects not one disease, but several[55]. How these distinctions impact on FDG-PET imaging is still evolving.

IMAGING PRIMARY GASTRIC CARCINOMA WITH FDG-PET

Unlike esophageal carcinoma, in which the majority of tumors (particularly T2-T4) are identified on FDG-PET imaging, the primary gastric lesion is less well imaged by FDG-PET. This has been demonstrated in several series with sensitivity for detection of gastric lesions ranging from 21% to 100%[57-65]. Specificity ranged from 78% to 100%. There are several factors that affect the sensitivity and specificity to detect a primary gastric carcinoma. Significantly, there is a variable and occasionally intense uptake of FDG of a physiological nature within the gastric wall[61,63,66]. FDG uptake may also correspond to acute inflammation such as superficial or erosive gastritis[67]. This leads to two disadvantages in the detection of gastric cancer. Firstly, an awareness of this phenomenon must exist in order to avoid a high number of false positive diagnoses. Conversely, over-awareness may lead to failure to detect weakly enhancing and diffuse malignant lesions.

TUMOR SIZE AND DEPTH (T STAGE) AND FDG-PET

Tumor size and T stage may influence the sensitivity of PET imaging in the detection of the primary gastric lesion. In one study, sensitivity was as low as 21% for detecting tumors < 30 mm in size, and increased to 76% for lesions over 30 mm[62]. Gastric cancer limited to the mucosa or submucosa (T1 lesions), are less likely to be detected by PET than more advanced T2-T4 lesions. Sensitivity for detection of early gastric cancers (T1) ranges from 26% to 63%, whereas that for more advanced disease (T2-T4) ranges from 83%-98%[57,61,62,65]. Figure 4 graphically depicts the range of sensitivity in diagnosis of early and advanced gastric cancer.

Histological subtype variants also influence glucose uptake and therefore the ability of PET to detect the primary lesion. The ability of FDG-PET to detect non-intestinal gastric primary tumors can range from 0% for T1 non-intestinal primaries to 77% for advanced non-intestinal disease. For intestinal type tumors, sensitivity ranges from 44% for T1 tumors to 92% for T2 or greater disease[62,63,68]. This may relate to the fact that the GLUT-1 transporter has been shown to be preferentially expressed on the intestinal type gastric carcinoma cell subtype, with decreased expression on mucous-secreting and signet ring type cells[69,70]. GLUT-1 expression has been shown in multivariate analysis to be the most influential factor relating to FDG uptake in gastric carcinoma, although the relationship between histological subtype and SUV uptake and sensitivity of FDG-PET has not been consistent across studies[71,59-61].

TECHNIQUES TO IMPROVE DETECTION OF THE PRIMARY GASTRIC LESION

Simple measures such as distention of the stomach by water or, less commonly, food have been shown to improve the accuracy of detection of gastric lesions both pre-operatively and in the post-operative remnant stomach[72-74]. In an effort to improve detection of gastric cancer by PET, the pyrimidine analog FLT has been used as an alternative radiotracer. One study demonstrated increased sensitivity of FLT-PET for detection of gastric tumors, especially if those tumors which were not FDG avid[58]. This may improve detection of previously difficult-to-detect tumor types such as mucin-producing and signet ring cell tumors. A second smaller study showed comparable efficacy between the two moieties[59]. In both studies, mean SUV uptake was lower for FLT-PET than for FDG-PET. Additional improvements may be made

Table 3 Gastric cancer lymph node staging by positron emission tomography

Ref.		*n*	Sensitivity (%) PET	Specificity (%) PET	Sensitivity (%) CT	Specificity (%) CT
Chen *et al*[57]		61	61	92	77	62
Kim *et al*[60]		73	40	95	71	71
Mochiki *et al*[61]		85	23	100	65	77
Mukai *et al*[62]		62	34.50	97	62.10	87.90
Yeung *et al*[64]		23	22	97		
Yoshioka *et al*[75]	Low resolution	42	47	62		
	High resolution	41	73	78		
Yun *et al*[65]		81	35	97	52	94
Tian *et al*[78]		38	60	100		
Yang *et al*[79] (PET-CT)		78	37	97.20	60.50	83.30

PET: Positron emission tomography; CT: Computed tomography.

possible by improving spatial resolution of the imaging equipment[75].

SCREENING FOR GASTRIC CARCINOMA WITH FDG-PET

FDG-PET has not been shown to be an effective screening tool for the diagnosis of gastric cancer. In one study, combined with endoscopy in asymptomatic individuals, PET-CT detected 2/20 cancers from 2861 patients screened giving a sensitivity of only 10% and a positive predictive value of 8.3%; 18/20 cancers were early gastric cancers (T1). There were 22 false positives on this study. There was no significant difference between the SUV values of the false positives and the true positives[76]. A second study of 1336 asymptomatic patients detected two gastric cancers in addition to nine other malignancies. The rate of false positive in this study was three times the rate of true positive findings[77]. Therefore, the screening sensitivity of FDG-PET in an asymptomatic population is less again than that in a diseased population.

FDG-PET AND LYMPH NODE STATUS: GASTRIC ADENOCARCINOMA

Survival in gastric cancer patients decreases with lymph node involvement, and with the number of lymph nodes involved. Knowledge of lymph node status therefore is not only of importance with respect to prognosis, but may also guide surgical treatment planning and which patients may benefit from neoadjuvant chemotherapy.

FDG-PET has been examined both alone, in comparison with CT imaging, and combined as CT-PET, in the preoperative assessment of the nodal status of gastric cancer (see Table 3). The sensitivity of PET is generally low for the detection of lymph node metastases, ranging from 22% to 60% for normal resolution scans[57,60-62,64,65,75,78,79]. It is possible that this may reflect the low spatial resolution of PET at 7 mm-9 mm which leads to difficulty discriminating perigastric lymph nodes from the gastric primary tumor, as sensitivity has been shown to increase to up to 73% with a higher resolution scan[75]. This compares poorly with the sensitivity of CT which ranges from 52% to 77% in the same series. By contrast the specificity of PET is higher than that of CT, ranging from 62%-100%, compared to CT (range, 62%-94%)

The sensitivity and specificity of PET are also influenced by lymph node staging status (i.e., N1, N2, or N3 nodal metastases). In three studies which stratified sensitivity by lymph node status, CT was significantly more sensitive for N1 disease[60,61,65], whereas similar levels of sensitivity and specificity were seen in N3 disease for both imaging modalities; however, this may have reflected the low prevalence of N3 disease in the study groups. Increased SUV of the primary tumor was correlated positively with lymph node metastases in two studies[57,61], possibly indicating increased glucose transport capacity which may in turn correlate with increased aggressiveness of the primary tumor[69].

PERITONEAL DISEASE

A common site of spread for gastric adenocarcinoma is the peritoneum. As many as 25% of patients with locally advanced tumors on EUS will have sub-radiographic occult peritoneal disease that may be identified only at laparoscopy[80]. PET is not a reliable indicator of peritoneal disease, with sensitivity for detection of peritoneal carcinomatosis of between 9% and 30% with normal resolution scans, and increased to 50% sensitivity with the use of a higher resolution 3.9 mm slice. This compares unfavorably with CT which demonstrates a sensitivity of 76%-80% for peritoneal cancer[57,75,81]. Peritoneal lesions are often small and diffuse in nature, which may go some way to explaining the low detection rate. Specificity remains high at 79%-98% in the same series, with less specificity with higher resolution imaging. Due to the need to confirm the absence of metastatic peritoneal spread prior to definitive surgery, staging laparoscopy may still be necessary, as this is the most sensitive modality to evaluate the peritoneum[82,83].

RESPONSE TO TREATMENT

With the introduction of neoadjuvant or perioperative

Table 4 Positron emission tomography computed tomography for the detection of gastric cancer recurrence

Author	Yr	*n*	Discriminating factor	Sensitivity (%) PET	Specificity (%) PET
De Potter *et al*[85]	2002	33		70	69
Jadvar *et al*[90]	2003	16		94	100
Yoshioka *et al*[75]	2003		Liver	78-85	82-74
			Lung	67	88
			Bone	30	82
			Pleural	4	100
			Ascites	24	76
Patriti *et al*[89]	2007	51		100	
Nakamoto *et al*[88]	2009	44	Previous suspicious imaging	80	100
		14	Tumor markers positive	73	83
		26	Routine	50	88
Park *et al*[117]	2009	105		75	77
Sim *et al*[86]	2009	52		68.40	71.40
Sohn *et al*[118]	2009	212	Post ablation	0	

PET: Positron emission tomography.

chemotherapy it is of interest to try to determine those who may respond to such chemotherapy, and those who are likely to fail to respond. This may be crucial in future in order to spare non-responders further potentially toxic chemotherapy, or to switch to another, non cross resistant regimen. The advantage of PET over CT in this regard is that the CT response by RECIST (Response Evaluation Criteria in Solid Tumors) as measured by the change in size may be a late manifestation of a response. PET may demonstrate a decrease in FDG uptake at an earlier stage than could be demonstrated by conventional imaging.

In one study of 44 pure gastric carcinoma patients treated with neoadjuvant cisplatin and 5-fluororuacil, 35 showed FDG uptake at baseline, before the initiation of chemotherapy. The PET response at 14 d post-chemotherapy was correlated with histopathological response at the time of surgery. The PET response was defined as > 35% reduction in the SUV value of the target lesion. A histopathological response was defined as < 10% viable tumor cells remaining in the operative surgical specimen. A metabolic response correctly predicted the histological response after completion of chemotherapy in 10/13 responding and 19/22 non-responding tumors, corresponding with a sensitivity of 77% (95% CI: 46%-95%) and a specificity of 86% (95% CI: 65%-97%)[41]. Metabolic response appeared to correlate significantly with survival. At 2-year follow-up, survival in the metabolic responder group was 90%, compared with 25% in the metabolic non responder group. A second smaller study in the setting of metastatic gastric cancer using chemotherapy and the biologic agent cetuximab demonstrated in this study, PET demonstrated a sensitivity of 83% and a specificity of 75% for the prediction of ultimate best response by RECIST. There was also a significant correlation between metabolic response and progression-free survival in this cohort[84].

FDG-PET AND PREDICTION OF PATIENT SURVIVAL: GASTRIC ADENOCARCINOMA

Data on survival with respect to PET-positive tumors may be confounded by the fact that PET-negative tumors in most studies may represent earlier stage disease. For example, in one study, The 2-year survival rate for patients with PET-positive cancers was 65.9%, and for those with PET-negative cancers was 94.4%, but a significant proportion of PET-negative tumors were T1/T2 *vs* T3/T4 for the tumors visible on PET[61]. One study on recurrent gastric carcinoma with 33 patients showed a higher median survival for those with PET negative recurrence *vs* PET positive recurrence of 18.5 mo *vs* 6.9 mo respectively, however, other studies have failed to corroborate this finding[63,85].

FDG-PET TO DETECT RECURRENCE OF RESECTED DISEASE: GASTRIC ADENOCARCINOMA

When compared to contrast CT, PET showed a non-significant trend towards decreased sensitivity and increased specificity in the detection of recurrent disease. Contrast-enhanced CT was significantly more sensitive for the diagnosis of peritoneal recurrence (87% *vs* 47%)[86]. This concurs with another series demonstrating a high sensitivity of 78% and 67% for liver and lung lesions, respectively, with a lower sensitivity of 30% for bone metastases. Sensitivity for pleural carcinomatosis and ascites were also similarly low[75]. As FDG also demonstrates uptake in acute inflammation and fractures in addition to physiological uptake in the abdomen, this may lead to false positives in the detection of boney disease[87]. Table 4 summarizes these data.

Notably, the utility of FGD-PET in the detection of recurrent gastric cancer is largely dependent on the prevalence of recurrent disease in the screened population. In a population undergoing routine screening examination following definitive primary therapy the sensitivity of screening may be as low as 50%-70%. In contrast, positive predictive value is high in a high prevalence population (i.e., those in whom disease is suspected). This is illustrated when comparing the positive predictive value of 100% in a population with a suspicion of disease based on previous radiological imaging *vs* 25% in a population with no clinical or radiological suspicion of recurrent disease[85,86,88]. If the population undergoing testing has an a priori suspicion of disease based on previous imaging or tumor markers, then sensitivity for detection may reach 94%-100%. Specificity is generally high at 70%-100% for PET in the detection of recurrent disease[89,90].

PANCREAS ADENOCARCINOMA

Pancreatic cancer ranks as one of the most lethal malignancies and only 20% are suitable for resection at presentation. Accurate delineation of tumoral extent and anatomy are crucial prior to surgery in order to avoid potentially futile laparotomy. Conventional work up includes abdominal ultrasound, CT, EUS and MRCP.

PET AND THE DIAGNOSIS AND MANAGEMENT OF PANCREATIC MALIGNANCY

As the normal pancreas exhibits low FDG uptake, and pancreatic tumors have been demonstrated to have high GLUT-1 expression, the expectation is that pancreatic tumors should not be difficult to differentiate from the normal parenchyma by FDG-PET[91]. In an initial study in 1997 by Zimny *et al*[92], 106 patients with pancreatic lesions were examined using FDG-PET; 85% of pancreatic carcinomas were correctly identified, and in 84% of cases of chronic pancreatitis it was possible to exclude malignancy. Ten of 11 false negatives were due to elevated plasma glucose. In patients with normal plasma glucose the sensitivity, specificity, positive and negative predictive values were 98%, 84%, 96% and 93%, respectively. The SUV of carcinoma was significantly higher than that of chronic pancreatitis (6.4 ± 3.6 for pancreatic carcinoma *vs* 3.6 ± 1.7 for chronic pancreatitis ($P < 0.001$). Inokuma *et al*[93] examined the utility of PET in the diagnosis of pancreas cancer in comparison to CT and EUS. In a study of 45 patients PET had a lower sensitivity than EUS, but a higher specificity than all other modalities, and highest positive predictive value and overall accuracy. In a larger study, comparing PET with CT and MRI, the sensitivity of PET was lower than that of CT but higher than that of MRI (91% CT *vs* 82% PET *vs* 78% MRI), and PET had the highest specificity and positive predictive value among the three modalities. There was no correlation between the SUV of the tumor and the degree of differentiation. The ability of PET to detect disease was improved by the correction of SUV for blood glucose[94]. The ability of PET to detect pancreatic cancer may be greater than CT at smaller lesion sizes[95]. In the differentiation of benign *vs* malignant cystic disease of the pancreas, Sperti *et al*[96] showed that PET was superior to CT with respect to sensitivity, specificity, positive and negative predictive value, and diagnostic accuracy at 94%, 94%, 89%, 97%, and 94%, respectively; these figures for CT were 65%, 88%, 73%, 83%, and 80%. A review by Gambhir *et al* suggested a sensitivity of 94% and a specificity of 90% for PET when compared with that of CT (84% and 75%, respectively)[97]

FDG-PET AND STAGING: PANCREAS ADENOCARCINOMA

FDG-PET is not the preferred modality to stage the depth of invasion or invasion of local-regional structures the primary tumor of the pancreas due to its poor spatial resolution. At this time, thin slice CT or EUS are better able to delineate the anatomical boundaries of the primary tumor and thus resectability. Similarly, PET is poorly sensitive for the detection of loco-regional lymph node metastases, which may be due to their proximity to the primary lesion. Sensitivity has ranged from as low as 49% to as high as 76% for the detection of local field lymph node involvement[98,99]. For pancreatic tumors, similar to gastric adenocarcinoma, FDG-PET is sensitive for the detection of metastatic disease to the liver and bone, but less so to the peritoneum. In a series of 168 patients Fröhlich *et al*[100] determined PET had a sensitivity of 97% for hepatic lesions > 1 cm, but only 43% for those < 1 cm, with 95% specificity. Three quarters of false positives were due to intrahepatic cholestasis. A study of 59 patients by Diederichs *et al*[99] confirmed these findings, with an overall sensitivity for the detection of hepatic metastases of 70%, again missing some metastases < 1 cm in diameter. The sensitivity for the detection of peritoneal disease was 25%.

IS SUV UPTAKE PROGNOSTIC IN PANCREATIC CANCER?

An SUV cut-off of ⩾ 4.0 was used by Sperti and colleagues to characterize patients with pancreatic cancer into two groups. Those with an SUV ⩾ 4.0 had an overall survival of only 7 mo, compared to 32 mo in the lower SUV group. This applied also to those who underwent resection. Tumor SUV was confirmed in multivariate analysis to be an independent predictor of survival[96]. This is in agreement with data published by Nakata *et al*[101] for patients with inoperable pancreatic tumors, in which those with a tumor SUV of > 3.0 were shown to have inferior survival to those with SUV uptake of < 3.0. In contrast to many other malignancies, proliferative activity as measured by the Ki67 index did not correlate with FDG uptake in pancreatic tumors[102].

PET AS A PREDICTOR OF RESPONSE TO CHEMOTHERAPY: PANCREAS ADENOCARCINOMA

PET has been used in an attempt to measure the response to neoadjuvant chemoradiotherapy in pancreatic cancer. In a study of 20 patients with locally advanced pancreas adenocarcinoma, of those who had > 50% reduction from the baseline SUV, 10% had a complete surgical resection, compared to 6% of those who had < 50% reduction. Those with a significant response also had a 23.2 mo survival compared to 11.3 mo in those who did not respond[103]. This is in agreement with a study by Bang *et al* which demonstrated the superiority of PET in the detection of a treatment response to chemoradiotherapy, detecting a response in one-third of patients, where conventional

CT failed to detect any response. Those who developed a response on PET also had significantly longer survival than those who did not. The PET and tumor marker response following palliative chemotherapy were also correlated positively with patient survival in a recent Japanese study[104] which contrasts with results of a study by Kobayashi *et al*[105] in which only a fall in tumor markers and not SUV was correlated with survival.

DETECTION OF RECURRENT DISEASE

Ruf *et al*[106], in a study of 31 patients with suspected recurrence after surgery, demonstrated that PET was superior to the combination of CT and MRI in the detection of recurrence (96% *vs* 39%). CT/MRI failed to detect any local recurrence, but did perform well in the detection of small hepatic metastases when compared to PET (92% *vs* 42%). Thus PET may be superior in the detection of recurrence within the tumor bed, but CT/MRI may have better discriminatory power within the hepatic parenchyma. PET may also complement the use of tumor markers or CT for the detection of recurrent disease when CT findings are equivocal, as demonstrated in a small study by Rose *et al*[95], where PET detected 100% of recurrences felt to be equivocal on CT. In a recent study of 45 patients with suspected recurrent disease, PET fused with contrast CT was shown to have a sensitivity of 94.7% for the detection histologically proven metastatic disease. Notably there was also a high sensitivity in this study for the detection of all sites of recurrence, with sensitivity for detection of local recurrence, abdominal lymph node metastasis, and peritoneal dissemination being 83.3%, 87.5%, and 83.3%, respectively[107].

METHODS OF IMPROVING THE ACCURACY OF PET IN PANCREAS CANCER

Although PET is superior to CT for the differentiation of benign *vs* malignant lesions, false positives may occur, most commonly due to pancreatitis, post instrumentation of the biliary tree, due to retroperitoneal fibrosis or hemorrhage or inflammation of a pancreatic pseudocyst. If C-reactive protein serum levels are elevated, the specificity of PET may fall to 50%[108]. Using delayed PET may aid in the differentiation of benign *vs* malignant lesions as evidenced in a prospective series of 47 patients where the diagnostic accuracy for malignant *vs* benign disease was 91.5% using this method[109]. Optimal glycemic control is also an important factor in the accuracy of PET scanning in pancreatic disease as noted in the study by Zimny where 91% of false negative results were due to hyperglycemia reducing the sensitivity of PET from 96% to 63% in those with an abnormally high serum glucose[92].

The fusion of PET-CT may show promise. A retrospective study by Lemke *et al*[110] showed that use of PET-CT improved the sensitivity of either individual imaging modality. Sensitivity was 76% for CT, 84% for PET and 89% for PET-CT but this came at a cost of a loss of specificity. Addition of CT imaging to fusion PET-CT may lead to further gains. In another study the sensitivity for the detection of metastatic disease by PET-CT, CT, and PET-CT plus CT was 61%, 57%, and 87%, respectively[111]. Enhanced PET-CT has also been shown to be superior to PET alone compared to unenhanced PET-CT imaging in two studies[107,112]. Use of the alternative radiotracer FLT has not been shown to be of benefit in pancreas cancer. In a small pilot study, FLT-PET demonstrated low levels of uptake in the primary tumor and detected only 40% of primary pancreatic tumors compared to 100% with FDG-PET[113].

CONCLUSION

FDG-PET imaging is now a standard practice in staging cancers of the esophagus. The role of FDG-PET/CT imaging in staging gastric carcinoma, however, is complicated by the higher rate of FDG-non-avid malignancies and by the false positive rate within the stomach due to inflammatory conditions. For each upper GI malignancy, depth of invasion and nodal status are not well evaluated by FDG-PET scans. However, for locally-advanced malignancies, an FDG-PET scan may be used to identify occult metastatic disease which may then significantly then change the treatment plan. A newer application of this imaging modality is the assessment of metabolic response, which correlates with chemotherapy sensitivity and survival. Preliminary prospective clinical studies suggest FDG-PET scans can predict response to therapy. With these data, the utility of FDG-PET scanning in upper GI malignancies is increasingly commonplace. With the identification of new FDG-PET tracers, we expect a further expansion of the application of PET imaging in upper GI malignancies.

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ORIGINAL ARTICLE

Osteopontin expression is associated with hepatopathologic changes in *Schistosoma japonicum* infected mice

Bo-Lin Chen, Gui-Ying Zhang, Wei-Jian Yuan, Shi-Ping Wang, Yue-Ming Shen, Lu Yan, Huan Gu, Jia Li

Bo-Lin Chen, Gui-Ying Zhang, Wei-Jian Yuan, Lu Yan, Huan Gu, Jia Li, Department of Gastroenterology, Xiangya Hospital, Central South University, Changsha 410008, Hunan Province, China
Shi-Ping Wang, Department of Pathogenic Biology, College of Xiangya Basic Medicine, Central South University, Changsha 410008, Hunan Province, China
Yue-Ming Shen, Department of Gastroenterology, Changsha Central Hospital, Changsha 410004, Hunan Province, China
Author contributions: Chen BL designed the research, performed the majority of the experiments, and wrote the manuscript; Zhang GY guided the research; Wang SP provided many facilities in animal modeling; Yuan WJ was involved in editing the manuscript; Shen YM, Yan L, Gu H, and Li J helped perform the research and participated in data analysis.
Supported by Grants from the National Natural Science Foundation of China, No. 81072038/H1617
Correspondence to: Gui-Ying Zhang, Dr., Department of Gastroenterology, Xiangya Hospital, Central South University, Changsha 410008, Hunan Province, China. guiyingzhang@hotmail.com
Telephone: +86-0731-84327282 Fax: +86-0731-84327321
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Abstract

AIM: To investigate osteopontin expression and its association with hepatopathologic changes in BALB/C mice infected with *Schistosoma japonicum*.

METHODS: The schistosomal hepatopathologic mouse model was established by abdominal infection with schistosomal cercaria. Liver samples were obtained from mice sacrificed at 6, 8, 10, 14, and 18 wk after infection. Liver histopathological changes were observed with hematoxylin-eosin and Masson trichrome staining. The expression of osteopontin was determined with immunohistochemistry, reverse transcription-polymerase chain reaction, and Western blotting. The expression of α-smooth muscle actin (α-SMA) and transforming growth factor-β1 (TGF-β1) were determined by immunohistochemistry. Correlations of osteopontin expression with other variables (α-SMA, TGF-β1, hepatopathologic features including granuloma formation and degree of liver fibrosis) were analyzed.

RESULTS: Typical schistosomal hepatopathologic changes were induced in the animals. Dynamic changes in the expression of osteopontin were observed at week 6. The expression increased, peaked at week 10 ($P < 0.01$), and then gradually decreased. Positive correlations between osteopontin expression and α-SMA ($r = 0.720$, $P < 0.01$), TGF-β1 ($r = 0.905$, $P < 0.01$), granuloma formation ($r = 0.875$, $P < 0.01$), and degree of liver fibrosis ($r = 0.858$, $P < 0.01$) were also observed.

CONCLUSION: Osteopontin may play an important role in schistosomal hepatopathology and may promote granuloma formation and liver fibrosis through an unexplored mechanism.

Key words: *Schistosoma japonicum*; Granuloma; Liver fibrosis; Osteopontin; BALB/C mice

Peer reviewers: Satoshi Mamori, MD, PhD, Department of Gastroenterology and Hepatology, Shinko Hospital, 1-4-47 Wakihama-cho, Chuo-ku, Kobe, Hyogo 651-0072, Japan; Hussein M Atta, MD, PhD, Professor, Department of Surgery, Faculty of Medicine, Minia University, Misr-Aswan Road, El-Minia 61519, Egypt

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INTRODUCTION

Schistosomiasis remains a huge threat to human health in tropical areas. Almost 12% of the population is in danger of this disease, with more than 200 million people infected annually[1]. In the past few decades, this disease has re-emerged and is still endemic in marsh, lake, and even mountainous regions of China, causing social hardship and economic burden[2]. It is now believed that the immune response of humans to schistosome eggs and the granulomatous responses they induce are the major causes of pathology in schistosomiasis[3]. The granulomas that form around the eggs impair blood flow in the liver and consequently induce portal hypertension[4]. On the other hand, the granulomas destroy the eggs and sequester or neutralize pathogenic egg antigens, leading to fibrosis in host tissues[5]. Once the immune response is activated, it is unlikely to be self-limiting, and schistosomiasis liver damage will continue, even after effective insecticide treatment[6]. Increasing reports of praziquantel treatment failures have highlighted the need for advanced knowledge of schistosomal hepatopathologic mechanisms and for new therapeutic strategies.

Osteopontin is granulomatogenic and has chemokine functions (mediating T lymphocyte and macrophage migration), cytokine activity (modulating T-helper 1 and 2 cytokine production), and several inflammatory and anti-inflammatory effects (regulation of nitric oxide generation)[7]. Recent work has demonstrated the important role osteopontin plays in mediating hepatic inflammation[8]. Upregulation of osteopontin expression early in the development of steatohepatitis, and its possible role in signaling the onset of liver injury and fibrosis in experimental nonalcoholic steatohepatitis have been reported[9].

These limited findings led us to hypothesize that osteopontin may be engaged in the immunopathogenesis of schistosomiasis liver damage. In our current study, we investigated the dynamic changes in osteopontin expression in *Schistosoma japonicum* (*S. japonicum*)-infected mouse liver. We also examined the relationship between osteopontin and hepatopathology and potential promoters of fibrosis progression such as hepatic stellate cells (HSCs) and transforming growth factor-β1 (TGF-β1) to obtain possible clues for further studies on the cellular and molecular mechanisms involved in schistosomal hepatopathology.

MATERIALS AND METHODS

Parasite and laboratory animals

Six-week-old BALB/C female mice were purchased from the Experimental Animal Center (Central South University, Changsha, Hunan, China). All animal experiments were performed in accordance with the Chinese Council on the Animal Care Guide for the Care and Use of Laboratory Animals. *Oncomelania hupensis* harboring *S. japonicum* cercariae were obtained from the Center for Schistosomiasis Control and Prevention (Yueyang, Hunan, China).

Animal treatment

One hundred BALB/C mice were randomly divided into the control group and the model group (n = 50 each). Mice in the model group were percutaneously infected with *S. japonicum* by placing a glass slide carrying 15 ± 1 cercariae in non-chlorine water on its abdomen for 20 min. Mice in the control group were treated with non-chlorine water containing no cercariae. All mice were kept at 20-25 ℃ in a 12-h light/12-h dark cycle with free access to food and water. At 6, 8, 10, 14, and 18 wk after infection, 10 mice from each group were randomly selected and sacrificed. Liver tissues were extracted and cut into two parts: the left lobes of the liver were fixed in 4% paraformaldehyde for 12 h; the remaining portion of the liver was preserved at -80 ℃ until use.

Histopathological study

Paraformaldehyde-fixed liver specimens were dehydrated in a graded alcohol series. Following xylene treatment, the specimens were embedded in paraffin blocks, cut into 5-μm thick sections, and placed on glass slides. The sections were then stained with hematoxylin and eosin (HE) and Masson trichrome (MT) according to standard procedures. To describe and evaluate liver pathological changes, a pathologist who was blinded to the research design examined 10 different low-power fields of HE- and MT-stained sections (selected fields were in almost the same location) for each mouse. In addition, the percentage of collagen calculated by a multimedia color image analysis system (Image-Pro Plus 6.0) was measured as a relative objective index (because a histological/fibrosis score that is evaluated by pathologists is susceptible to the ability and subjective judgment of the pathologist) to evaluate the degree of liver fibrosis. Each MT-stained section was examined at 100 × magnification. Every field analyzed contained a granuloma, portal area, or a centrilobular vein. Fibrotic areas were scanned and summed by the software. The percentage of collagen was expressed as the ratio of the collagen-containing area to the whole area, and the result was determined as the mean of ten different fields of each section. Furthermore, the granuloma dimension was also measured at a magnification of 100× using an ocular micrometer. Only non-confluent granulomas containing eggs in their centers were measured[10]. Granuloma dimension = maximum width × maximum length. Mean granuloma dimension of each section = sum of all granuloma dimensions in each section/number of granuloma in each section.

Immunohistochemistry

Immunohistochemical staining was performed with the PV-6001/6002 Two-Step IHC Detection Reagent (ZSGB-BIO, China). The sections were dewaxed, dehydrated, immersed in citrate buffer (0.01 mol/L, pH 6.0), heated at 100 ℃ in a microwave oven 6 × 2 min, incubated in 3% H_2O_2 in deionized water for 10 min to block endogenous peroxidase activity, and washed 2 × 3 min with phosphate-buffered saline (PBS). The sections were

then incubated overnight at 4 ℃ with antibodies against osteopontin (mouse monoclonal; 1:300; Santa Cruz Biotechnology, United States), α-SMA (mouse monoclonal; 1:300; Santa Cruz Biotechnology), and TGF-β1 (rabbit polyclonal; 1:300; Santa Cruz Biotechnology). After washing 2 × 3 min with PBS, the appropriate second antibody was added to the sections and incubated at 37 ℃ for 30 min. Then, the sections were washed 2 × 3 min with PBS, and the color was developed with diaminobenzidine (DAB) for about 5 min. Nuclei were lightly counterstained with hematoxylin. Negative controls included incubation with PBS without the primary antibody. The integral optical density (IOD) was measured with Image-Pro Plus 6.0, and the result was determined as the sum of five different fields (one in the center and four in the periphery) of each section. The IOD of the target protein was defined as the sum of the optical densities of all the positive pixels in the image, which represents the quantity of the targeted protein. The IOD is considered to be more accurate than average optical density as it considers both the intensity and area.

Reverse transcription-polymerase chain reaction

Total RNA was extracted from frozen liver tissue with TRIZOL Reagent (Invitrogen, United States). Complementary DNA (cDNA) was synthesized from total RNA using a ReverTra Ace-α-TM First Strand cDNA Synthesis kit (Toyobo, Japan). Relative quantification of target gene expression was performed using the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The primer sequences were osteopontin forward 5'-CCAGGTTTCTGATGAACAGT-3' and reverse 5'-GTGTGTTTCCAGACTTGGTT-3', which yielded a fragment of 193 bp, and GAPDH forward 5'-AACTTTGGCATTGTGGAAGG-3' and reverse 5'-GGATGCAGGGATGATGTTCT-3', which yielded a fragment of 132 bp. For the first step, the following components were mixed to obtain the specified concentrations in a final 20 μL reaction volume: 1 μL denatured total RNA (1 μg/μL), 4 μL 5 × buffer, 2 μL dNTP mixture (10 mmol/L), 1 μL RNase inhibitor (10 U/μL), 10 μL RNase-free H_2O, 1 μL Oligo $(dT)_{20}$ (10 pmol/μL), and 1 μL ReverTra Ace. The reaction was performed at 42 ℃ for 20 min, followed by 99 ℃ for 5 min, and 4 ℃ for 5 min. In the second step, 1 μL cDNA was mixed with 0.5 μL each sense and anti-sense primers (100 μmol/L each), 2 μL dNTP mixture (2 mmol/L), 1.5 μL $MgCl_2$ (25 mmol/L), 2 μL 10 × polymerase chain reaction (PCR) buffer, 0.5 μL Taq DNA Polymerase (500 U), and 12 μL PCR H_2O. PCR was performed as follows: denaturation at 95 ℃ for 5 min; 32 cycles of denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s, and elongation at 72 ℃ for 30 s; and final elongation at 72 ℃ for 5 min. The PCR products were separated by electrophoresis on 1.5% agarose gels (sample volume: 10 μL, voltage: 120 V) and visualized with ethidium bromide staining and ultraviolet illumination. We used gel OD analysis software (Gel-Pro 4.0) to scan and calculate the IOD of strips. The relative mRNA expression of osteopontin was represented as the ratio of osteopontin:IOD and GAPDH:IOD.

Western blotting

Frozen tissue specimens (500 mg) were homogenized on ice in 1 mL lysate prepared from a Total Protein Extraction kit (ProMab, United States) and then ultrasonicated for 3 × 3 s. The crude protein fractions were obtained by centrifuging the homogenates at 9000 × *g* for 10 min at 4 ℃. The supernatant was used as the protein fraction. Gel samples were prepared by mixing protein samples with sample buffer and boiling at 100 ℃ for 3 min. Nuclear and cytoplasmic proteins were separated with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis in running buffer. After electrophoresis, the proteins were transferred to nitrocellulose membrane (Pierce, United States) in transfer buffer at 300 mA constant current for 70 min on ice. Non-specific binding sites were blocked by incubating in PBS containing 5% nonfat milk for 2 h at 37 ℃. Membranes were then incubated with primary antibodies (mouse osteopontin monoclonal; 1:500; Santa Cruz Biotechnology and mouse monoclonal GAPDH; 1:1000; ProMab, United States) overnight at 4 ℃. The membranes were then washed 5 × 4 min with PBS-Tween 20 (PBST) and incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG; 1:50 000; Zymed, United States) for 1 h at 37 ℃. After the membranes were washed 5 × 4 min in PBST, enhanced chemiluminescence detection of the target protein was performed. The film was scanned, and the image was analyzed with Gel-Pro 4.0. The relative levels of osteopontin were represented as the ratio of osteopontin:IOD and GAPDH:IOD.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software. Data were expressed as mean ± SD. A normality test was performed before statistical analysis. Comparisons between groups and time points were performed using one-way analysis of variation (homogeneity of variance: S-N-K; heterogeneity of variance: Tamhane). Correlation analysis was performed with linear regression. *P* values less than 0.01 (heterogeneity of variance) or 0.05 were considered statistically significant.

RESULTS

Schistosomal hepatopathology

Both HE and MT staining revealed a parallel change over time (Figure 1, left and middle). The control group showed normal hepatocyte morphology (Figure 1A), but the model group showed typical hepatopathological characteristics of schistosomiasis with remarkable acute granuloma formation and subsequent liver fibrosis from week 6 through week 18 (Figure 1B-F). At week 6, inflammatory cells had infiltrated around the schistosome eggs and formed granulomas, which were mainly distributed in portal areas; collagen fibers were only interspersed among the periphery of the granulomas (Figure 1B). The granuloma size and the quantity of inflammatory

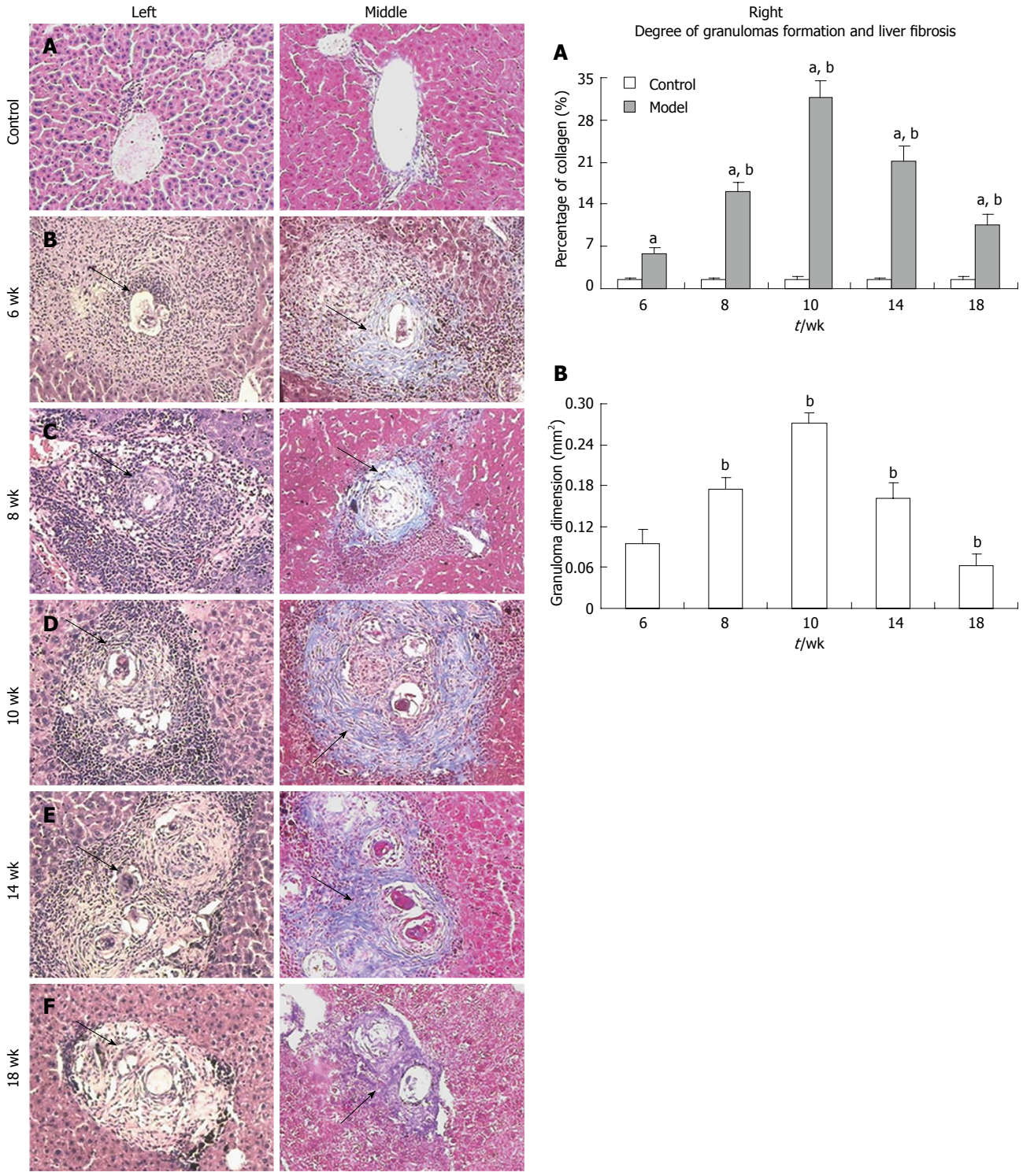

Figure 1 Representative images of hepatopathological changes over time. HE (left) and MT (middle) staining and data showing the degree of granuloma formation and liver fibrosis (right). Arrows show granulomas. Collagen fibers are stained blue (MT staining). 100 × original magnification. HE: Hematoxylin and eosin; MT: Masson trichrome. [a]$P < 0.05$ *vs* control; [b]$P < 0.05$ *vs* previous.

cells increased at week 8. Numerous fibrocytes appeared at the periphery of the lesions, and the collagen fibers became longer and thicker (Figure 1C). The granulomas reached their peak in size and quantity at week 10 (Figure 1D). Numerous inflammatory cells such as neutrophils, lymphocytes, and eosinophils were seen infiltrating in the granulomas, and numerous collagen fibers were wrapped or stretched into the interior of the granulomas. Some fibers extended from portal areas or inflammatory lesions to the lobule, which had been cut apart and re-built. In some serious cases, pseudolobules formed. At week 14, fibrocytes and collagen fibers eventually became the predominant feature of granulomas and formed typical chronic granulomas, whereas other cell types decreased in

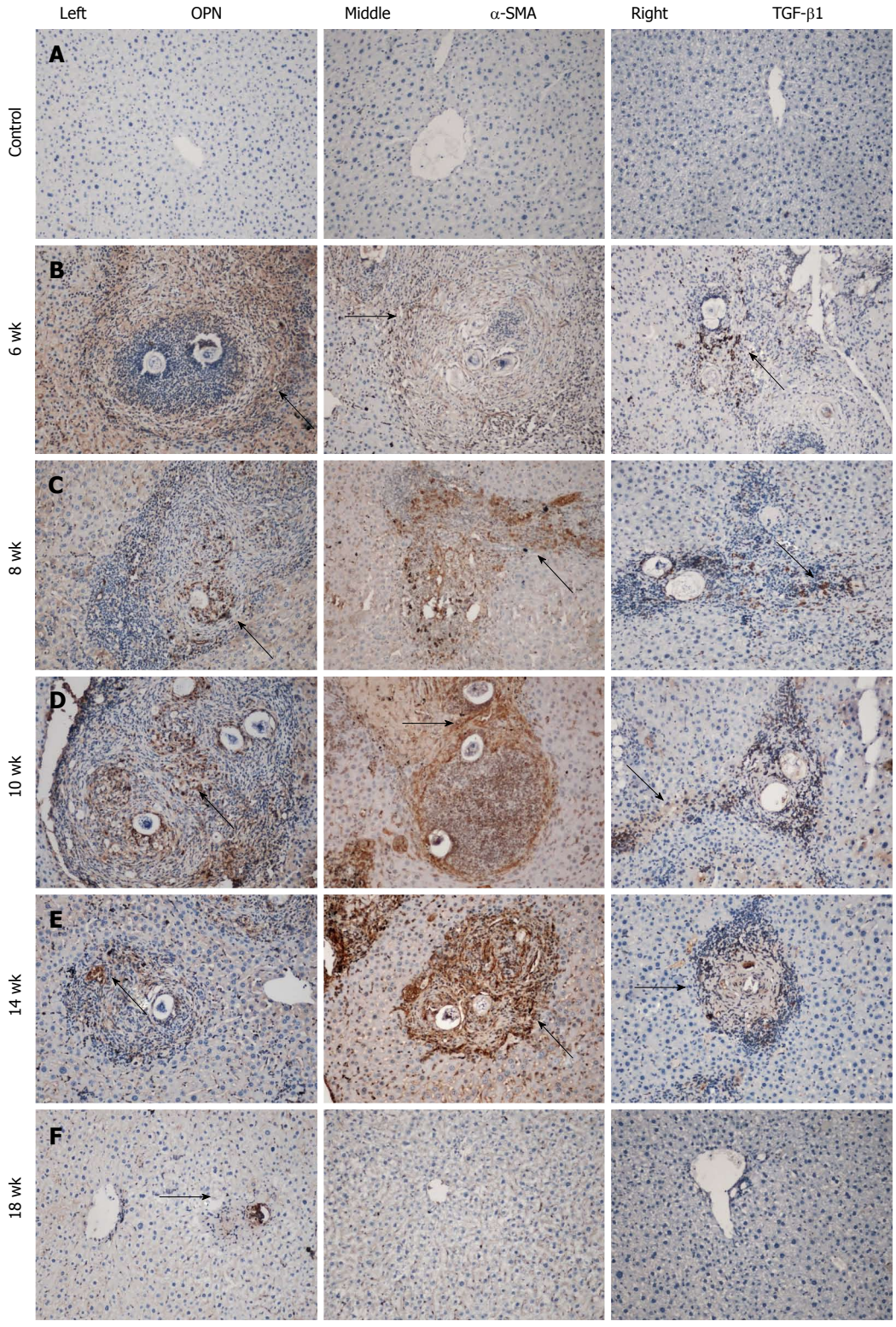

Figure 2 Representative images of immunostaining for osteopontin, α-SMA, and TGF-β1 over time in mouse liver. Positive staining is yellow brown, and arrowheads show positive cells. 100 × original magnification. OPN: Osteopontin; SMA: Smooth muscle actin; TGF: Transforming growth factor.

number (Figure 1E). The schistosome eggs degenerated and disintegrated at week 18, and fibrosis was obviously reduced but more stable (Figure 1F). The collagen percentage and the granuloma dimension in each group also showed a similar change over time (Figure 1, graphs A and B, right).

Expression of osteopontin, α-SMA, and TGF-β1 with immunohistochemistry

Immunohistochemistry for osteopontin, α-SMA, and TGF-β1 demonstrated a similar change that paralleled the development of hepatopathology over time (Figure 2A-F, left to right). Few if any, scarcely distributed cells with

Table 1 Integral optical density of immunostaining in the groups over time

Groups	Staining	Week 6	Week 8	Week 10	Week 14	Week 18
Control group IOD	Osteopontin (× 10^2)	0.52 ± 0.06	0.55 ± 0.07	0.56 ± 0.06	0.50 ± 0.06	0.50 ± 0.06
	α-SMA (× 10^2)[3]	0	0	0	0	0
	TGF-β1 (× 10^3)	0.20 ± 0.02	0.21 ± 0.02	0.20 ± 0.02	0.20 ± 0.03	0.20 ± 0.02
Model group IOD	Osteopontin (× 10^2)	6.45 ± 0.54[1,2]	10.21 ± 0.80[1,2]	31.20 ± 2.83[1,2]	6.00 ± 0.54[1,2]	2.26 ± 0.28[1,2]
	α-SMA (× 10^2)	0.93 ± 0.09[2]	18.19 ± 1.62[2]	39.13 ± 4.37[2]	30.93 ± 3.87[2]	1.45 ± 0.16[2]
	TGF-β1 (× 10^3)	2.52 ± 0.24[1,2]	4.90 ± 0.50[1,2]	9.20 ± 1.25[1,2]	3.84 ± 0.36[1,2]	0.19 ± 0.02[2]

[1]Compared with control group: $P < 0.01$; [2]Compared with previous time point: $P < 0.01$; [3]The staining of α-SMA in the control group was measured, and the result was zero. Results are expressed as the mean IOD (× 10^2 or × 10^3) ± SD. SMA: Smooth muscle actin; IOD: Integral optical density.

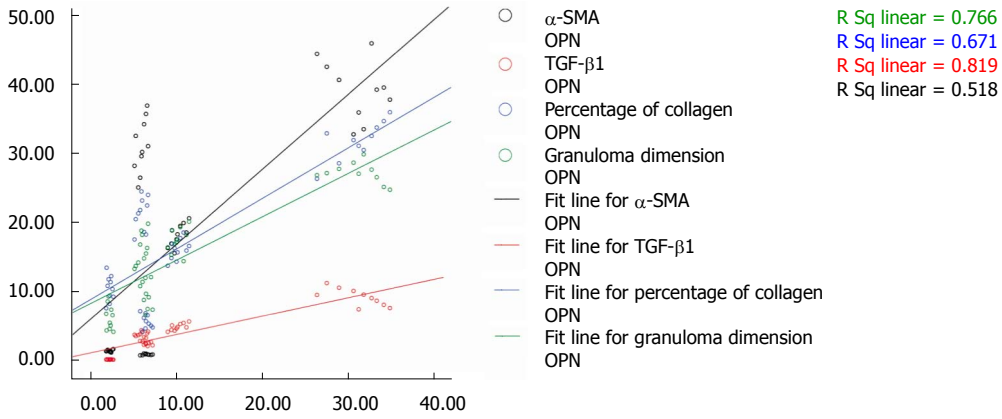

Figure 3 Correlation of osteopontin mRNA expression with α-SMA, TGF-β1, percentage of collagen, and the granuloma dimensions in the model group. x-axis: IOD of osteopontin (× 10^2); y-axis: Black line: IOD of α-SMA (× 10^2); Red line: IOD of TGF-β1 (× 10^3); Blue line: Collagen (%); Green line: Granuloma dimension, mm^2 (× 10^2). OPN: Osteopontin; SMA: Smooth muscle actin; TGF: Transforming growth factor.

faint staining were seen in the normal control group (Figure 2A, left to right) throughout the experiment. At week 6 in the model group, positively stained cells were widely distributed in the area of inflammatory cell infiltration, which formed acute granulomas (Figure 2B, left to right). Strongly upregulated expression of these proteins was seen from week 8 to week 10. Many densely stained positive cells surrounded and infiltrated into the egg granulomas, accumulated in fibrotic areas, and stretched along the fibrous septum (Figure 2C and D, left to right). At week 14, there were still many positive cells distributed in the fibrotic granulomas and dispersed at the periphery of the granulomas. However, the expression weakened and returned to near normal levels at week 18 (Figure 2E and F, left to right). The IODs of immunostaining of individual proteins in the groups over time are shown in Table 1.

Correlation analysis

Correlation analysis revealed that the expression of osteopontin was positively correlated with expression of α-SMA and TGF-β1, and with hepatopathological changes (Figure 3). The R-square values were 0.720, 0.905, 0.815, and 0.875 for osteopontin expression with α-SMA, TGF-β1, percentage of collagen, and granuloma dimension, respectively ($P < 0.01$), implying that the expression of osteopontin may play an important role in the development of liver damage in *S. japonicum*-infected mice in this study.

Expression of osteopontin mRNA (RT-PCR) and protein (Western blotting)

Consistent with the above results, parallel changes were seen in the expression of osteopontin mRNA and protein (Figure 4). The control group showed no changes in expression throughout the experiment, but the expression levels of both osteopontin mRNA and protein in the model group were upregulated at week 6, reached a peak at week 8 ($P < 0.05$), and decreased gradually. Expression levels remained higher than those in the control group ($P < 0.05$).

DISCUSSION

Osteopontin is considered a strong chemoattractant and proinflammatory molecule that is involved in a wide range of physiologic and pathologic events, including angiogenesis, tumor metastasis, wound healing, tissue remodeling, and fibrosis[11-13]. Osteopontin is believed to constitute the central pathway of HSC activation, a key step in the development of hepatic fibrosis[14,15]. However, the role of osteopontin in schistosomal liver damage should be explored further.

In our current study, typical hepatopathological changes were induced in *S. japonicum*-infected animals. Over time during the experiment, the infected liver showed granuloma formation and obvious fibrosis that appeared

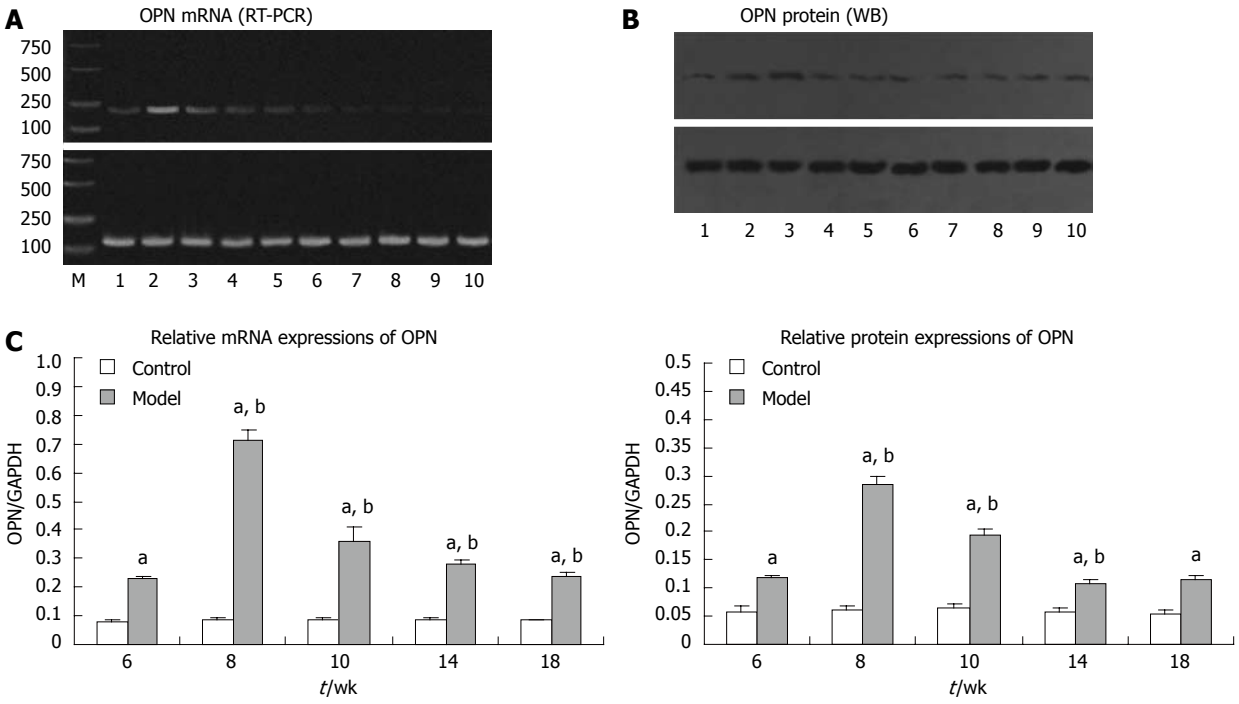

Figure 4 Profiles of osteopontin mRNA (RT-PCR) and protein (Western blotting) expression. No. 1-5 represent the model group at week 6, 8, 10, 14, and 18, respectively; No. 6-10 represent the control group at week 6, 8, 10, 14, and 18, respectively. A: Expression of osteopontin and GAPDH mRNA over time, the 100-bp GAPDH mRNA fragment was used as an internal control; B: Expression of osteopontin and GAPDH protein over time, the 37-kDa GAPDH band was used as an internal control; C: The IOD of osteopontin/GAPDH was expressed as the mean ± SD. M: Marker; n = 10 at each time. OPN: Osteopontin; RT-PCR: Reverse transcription polymerase chain reaction; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IOD: Integral optical density. $^{a}P < 0.05$ *vs* control; $^{b}P < 0.05$ *vs* previous.

at week 6 and peaked from week 8 to 14. Accompanying this pathological change, osteopontin expression (mRNA and protein) was highly upregulated and showed a strong correlation with pathologic changes in the liver and with the immunohistochemistry profiles of α-SMA and TGF-β1. Thus, our data imply involvement of osteopontin in the development of schistosomal hepatopathologic changes.

Determining the mechanisms and the detailed biological role of osteopontin in the process of schistosomal hepatopathology is beyond the scope of our current experiment, but other reports suggest some possibilities. Niki *et al*[16] reported that α-SMA is significantly increased following HSC activation and is considered a marker for HSC activation. TGF-β1 is not only upregulated following HSC activation, but also directly activates HSCs through the TGF-β1/Smad signal transduction pathway, leading to hepatic fibrosis[17]. The upregulated expression of both α-SMA and TGF-β1 and their positive correlation with osteopontin expression observed in this study add more evidence supporting their important role in the process of schistosomal hepatopathology and liver fibrosis.

In summary, typical schistosomal hepatopathological changes occurred during this experiment. The development of hepatopathology, including granuloma formation and liver fibrosis, was accompanied by dynamic expression of osteopontin, which correlated well with the expression of both α-SMA and TGF-β1 over time. Thus, osteopontin may play a key role in schistosomal hepatopathology, the mechanisms of which will require additional studies.

COMMENTS

Background

There are currently few effective therapies for *Schistosoma japonicum* (*S. japonicum*)-infected patients due to a lack of understanding of appropriate intervention targets. Further studies on new cellular and molecular mechanisms are urgently needed as the global *S. japonicum* epidemic is becoming more serious.

Research frontiers

Osteopontin, which is a chemoattractant and proinflammatory molecule, has been shown to be involved in tissue injury and remodeling in other diseases. However, its roles in schistosomal liver damage have yet to be explored.

Innovations and breakthroughs

This study first reports the dynamic changes in osteopontin expression (mRNA and protein) and its associations with pathologic changes in the liver of BALB/C mice infected with *S. japonicum*. This study analyzes the correlations of osteopontin expression with α-SMA and TGF-β1 expression and hepatopathologic features including granuloma formation and liver fibrosis.

Applications

By increasing our understanding of whether and how osteopontin is involved in schistosomal liver damage, this study may provide clues for further studies on the cellular and molecular mechanisms of schistosomal hepatopathology.

Peer review

The study is novel and the methodology is good.

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BRIEF ARTICLE

Hepatocellular carcinoma in cirrhotic patients with portal hypertension: Is liver resection always contraindicated?

Andrea Ruzzenente, Alessandro Valdegamberi, Tommaso Campagnaro, Simone Conci, Silvia Pachera, Calogero Iacono, Alfredo Guglielmi

Andrea Ruzzenente, Alessandro Valdegamberi, Tommaso Campagnaro, Simone Conci, Silvia Pachera, Calogero Iacono, Alfredo Guglielmi, Department of Surgery, Division of General Surgery "A", GB Rossi Hospital, University of Verona, 37134 Verona, Italy

Author contributions: Guglielmi A, Iacono C and Ruzzenente A designed the research and supervised this work; Valdegamberi A and Campagnaro T wrote the paper; Conci S and Pachera S performed the research; Ruzzenente A analyzed the data.

Correspondence to: Andrea Ruzzenente, MD, Department of Surgery, Division of General Surgery "A", GB Rossi Hospital, University of Verona, 37134 Verona, Italy. andrea.ruzzenente@fastwebnet.it

Telephone: +39-45-8124941 Fax: +39-45-585612

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Abstract

AIM: To analyze the outcome of hepatocellular carcinoma (HCC) resection in cirrhosis patients, related to presence of portal hypertension (PH) and extent of hepatectomy.

METHODS: A retrospective analysis of 135 patients with HCC on a background of cirrhosis was submitted to curative liver resection.

RESULTS: PH was present in 44 (32.5%) patients. Overall mortality and morbidity were 2.2% and 33.7%, respectively. Median survival time in patients with or without PH was 31.6 and 65.1 mo, respectively (P = 0.047); in the subgroup with Child-Pugh class A cirrhosis, median survival was 65.1 mo and 60.5 mo, respectively (P = 0.257). Survival for patients submitted to limited liver resection was not significantly different in presence or absence of PH. Conversely, median survival for patients after resection of 2 or more segments with or without PH was 64.4 mo and 163.9 mo, respectively (P = 0.035).

CONCLUSION: PH is not an absolute contraindication to liver resection in Child-Pugh class A cirrhotic patients, but resection of 2 or more segments should not be recommended in patients with PH.

Key words: Liver surgery; Hepatic resection; Hepatocellular carcinoma; Portal hypertension

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer death worldwide[1-3]. Surgical treatment is an effective treatment for HCC and the mortality after surgery has decreased in recent years in relation to improved surgical techniques and peri-operative management of patients[4-6]. The overall survival at 5 years after liver resection varies from 33% to 69% according to recent surgical series, although recurrence is still the major issue after surgery[7-13].

The indications for surgical resection depend on the characteristics of the tumor (stage, number of nodules, size, presence of vascular invasion), on the general condition of patient, and on liver functional reserve[14].

The presence of liver cirrhosis is the most important risk factor for the development of HCC, as 85%-95% of HCCs arise in cirrhotic livers[15-17]. HBV and HCV infections and alcohol abuse are the most frequent causes of cirrhosis: about 80%-85% of cases[7]. Portal hypertension (PH) is related to an increase in intrahepatic resistance due to the structural subversion of the liver and loss of vascular bed, and bleeding from gastroesophageal varices is one of the most important complications of cirrhosis[18]. On the basis of several studies[19,20], the American Association for the Study of Liver Diseases (AASLD)[21] defined as clinical PH the presence of esophageal varices or thrombocytopenia (platelet count < 100 000/mm^3) associated with splenomegaly. The European Association for the Study of the Liver (EASL)/AASLD guidelines consider PH as a relative contraindication to liver resection, because of the high risk of postoperative liver failure, as reported in some clinical series[21,22]. These results, however, have not been confirmed in more recent clinical studies[23-25].

The aims of this study are to assess the results of liver resection in patients with HCC and cirrhosis with PH and the relationship in terms of survival between Child-Pugh stage, the extent of hepatic resection and the presence of clinical PH.

MATERIALS AND METHODS

We retrospectively analyzed clinical data of 135 patients with cirrhosis undergoing liver resection with radical intent for HCC from 1995 to 2008 at the single Surgical Division of the Department of Surgery of the University of Verona. The patients' liver function was assessed by Child-Pugh classification. Clinical PH was defined according to AASLD guidelines as the presence of esophageal varices or thrombocytopenia (platelet count < 100 000/mm^3) associated with splenomegaly[21]. The extent of resection was defined according to the classification of Brisbane[26]. After liver resection, patients underwent follow-up with serum α-fetoprotein levels and abdominal ultrasound every 6 mo and computed tomography scan every 12 mo. The mean follow-up after surgery was 38.3 mo.

Statistical analysis

Data were collected and analyzed with SPSS statistical software (SPSS version 16.0 Inc., Chicago Ⅲ). The differences between categorical variables were analyzed with a χ^2 test. The differences between continuous variables were also analyzed with a χ^2 test.

Survival analysis was carried out with the Kaplan-Meier method. Univariate analysis for survival was performed with the Kaplan Meier method, with the Log Rank test to verify significance of differences. The statistical analysis included two different steps; in the first we analyzed the prognostic significance of the PH in all patients and in the second step we analyzed the prognostic significance of PH in different subgroups according to the Child-Pugh class and the extent of liver resection (wedge/segmentectomy or ⩾ 2 segments). Finally, multivariate analysis with Cox's regression model was performed with the following variables: Child-Pugh class, PH and type of hepatectomy. A *P* value lower than 0.05 was considered significant.

RESULTS

The prevalence of PH in all patients was 32.5%. The analysis of our data showed that patients with PH who underwent surgery had worse liver function compared to those without PH (patients in Child-Pugh B class 33% *vs* 11% respectively, $P < 0.01$), with serum bilirubin level > 2 mg/dL in 29% *vs* 3% respectively, $P < 0.01$ and serum transaminases AST > 80 U/L in 52% *vs* 25%, $P = 0.01$ and ALT > 80 U/L in 48% *vs* 19% respectively, $P = 0.01$ (Table 1). The one and 3-mo mortality rates were 4.6% and 13.9% and 1.1% and 3.3% for patients with and without PH, respectively ($P = 0.20$ and $P = 0.05$). The morbidity rate reached no statistical significance for patients with and without PH respectively (37% *vs* 32%, $P = 0.59$). The liver-related morbidity (ascites, encephalopathy, jaundice) was significantly higher in patients with PH than in patients without PH, 32% *vs* 13% respectively ($P = 0.03$) (Table 2).

The 3-year and 5-year survival in patients without PH was higher than in patients with PH (68.4% and 61.2% *vs* 48.7% and 44.9% respectively, $P = 0.047$). These results are reported in Figure 1A.

Survival analysis in patients with Child-Pugh B cirrhosis did not demonstrate significant differences in patients with or without PH (3-year survival of 31.3% *vs* 11.9%, $P = 0.465$) (Table 3).

Also, in Child-Pugh class A patients the survival analysis did not show significant differences in patients with or without PH, with a 3-year and 5-year survival of 63.0% and 57.3% *vs* 72.0% and 63.2% respectively ($P = 0.257$). These results are summarized in Table 3.

Furthermore, we evaluated the relationship between survival, extent of liver resection and PH in Child-Pugh class A patients.

In limited resections (wedge or one segment) we found no statistical differences between patients with or without PH. In these patients the 5-year survival was 72.4% and 61.4% respectively ($P = 0.458$, Figure 1B, Table 3).

When resection of two or more segments was performed, survival was significantly longer in patients without PH with a 5-year survival of 64.5% compared to 25.0% in patients with PH respectively ($P = 0.035$, Figure 1C, Table 3).

Multivariate analysis with Cox's regression model confirmed that Child-Pugh class was related to survival with a HR of 2.57 ($P < 0.01$), whereas PH and type of hepatectomy were not related to survival (Table 4).

DISCUSSION

Liver resection is currently the treatment of choice for

Table 1 Patients characteristics according to the presence/absence of portal hypertension

Variable	Portal hypertension		*P* value
	Yes (%)	No (%)	
n	44	91	
Age (yr)			0.55
< 70	31 (70)	60 (66)	
> 70	13 (30)	31 (34)	
Etiology of liver disease			0.21
Alcohol	7 (16)	24 (26)	
Viral hepatitis	36 (82)	59 (65)	
Other	1 (2)	8 (9)	
Serum ALT level (U/L)			0.01
< 80	23 (52)	74 (81)	
> 80	21 (48)	17 (19)	
Serum AST level (U/L)			0.01
< 80	21 (48)	68 (75)	
> 80	23 (52)	23 (25)	
Child-Pugh class			0.01
Class A	29 (66)	81 (89)	
Class B	15 (33)	10 (11)	
Bilirubin level (mg/dL)			0.01
< 2	31 (71)	88 (97)	
2-3	8 (18)	3 (3)	
> 3	5 (11)	0 (0)	
Albumin level (g/L)			0.01
< 28	7 (16)	8 (9)	
28-35	18 (41)	14 (15)	
> 35	19 (43)	69 (76)	
Platelet count			0.01
⩽ 100 000/mm^3	31 (70)	0	
> 100 000/mm^3	13 (30)	91 (100)	
Esophageal varices			0.01
Yes	17 (39)	0	
No	27 (61)	91 (100)	
α-fetoprotein level (ng/dL)			0.86
< 20	19 (43)	42 (46)	
> 20	25 (57)	49 (54)	
Size (cm)			0.03
< 3	14 (32)	30 (33)	
3-5	21 (48)	23 (25)	
> 5	9 (20)	38 (42)	
Number of nodules			0.51
Single	32 (73)	65 (72)	
2 HCC	8 (18)	12 (13)	
3 HCC or more	4 (9)	14 (15)	
Macrovascular Invasion			0.16
No	42 (95)	73 (80)	
Yes	2 (5)	18 (20)	
Microvascular invasion			0.24
No	29 (66)	43 (47)	
Yes	15 (34)	48 (53)	
Type of hepatectomy			0.58
Wedge resection	10 (23)	16 (18)	
Segmentectomy	26 (59)	52 (57)	
More than 1 segment	8 (18)	23 (25)	

ALT: Alanine transaminase; AST: Aspartate transaminase; HCC: Hepatocellular carcinoma.

single HCC and it is a safe treatment in terms of peri-operative complications, even in patients with liver cirrhosis[6,27]. The outcome of surgical resection is strongly related to hepatic functional reserve. For this reason, the majority of patients with liver cirrhosis cannot undergo surgery because of the high risk of postoperative liver failure.

Table 2 Mortality and morbidity rates according to the presence/absence of portal hypertension

	Portal hypertension		*P* value
	Yes (%)	No (%)	
n	44	91	
1 mo mortality	2 (4.6)	1 (1.1)	0.20
3 mo mortality	6 (13.6)	3 (3.3)	0.05
Overall morbidity	16 (37)	29 (32)	0.59
Cardiac complications	0	3 (3.3)	0.20
Pulmonary complications	10 (23)	18 (20)	0.71
Hepatic complications	14 (32)	12 (13)	0.03

Table 3 Survival analysis according to different groups of patient and presence/absence of portal hypertension

Variable	*n*	Median survival (mo, 95% CI)	Survival		*P* value
			3-yr	5-yr	
Overall					0.047
Without PH	91	65.1 (49.7-80.4)	68.4	61.2	
With PH	44	31.6 (3.4-59.9)	48.7	44.9	
Child-Pugh B					0.465
Without PH	10	27.7 (1.3-65.5)	31.3	31.3	
With PH	15	15.1 (11.7-18.5)	11.9	-	
Child-Pugh A					0.257
Without PH	81	65.1 (47.7-82.4)	72.0	63.2	
With PH	29	60.5 (6.4-114.6)	63.0	57.3	
CP A-limited Hx					0.458
Without PH	58	64.9 (62.9-67.0)	72.4	61.4	
With PH	21	94.0 (54.0-134.0)	72.7	72.7	
CP A-Hx ⩾ 2 segments					0.035
Without PH	23	163.9 (-)	64.5	64.5	
With PH	8	64.4 (54.0-134.0)	50.0	25.0	

CI: Confidence interval; PH: Portal hypertension; CP: Child-Pugh score; Hx: Hepatic resection.

Table 4 Multivariate Cox's regression model of variables related to survival

Variable	HR	95% CI	*P* value
Child-Pugh class (B *vs* A)	2.57	1.31-5.01	0.005
Type of hepatectomy (⩾ 2 segments *vs* limited)	1.05	0.50-2.21	0.880
PH (presence *vs* absence)	1.51	0.84-2.68	0.160

CI: Confidence interval; PH: Portal hypertension.

PH in cirrhotic patients is considered a relative contraindication for surgery in EASL/AASLD guidelines. Bruix *et al*[12] analyzed the outcome of 29 Child-Pugh class A patients with PH [defined as porto-hepatic gradient (HVPG) greater than 10 mm Hg] and observed a higher likelihood of postoperative liver failure in these patients compared to those without PH. The authors justified these results because liver resection in patients with PH can reduce the portal vascular bed without a reduction of portal flow and this condition can lead to a further increase of portal pressure. These hypotheses were not confirmed by a study by Fujisaki *et al*[28] that reported

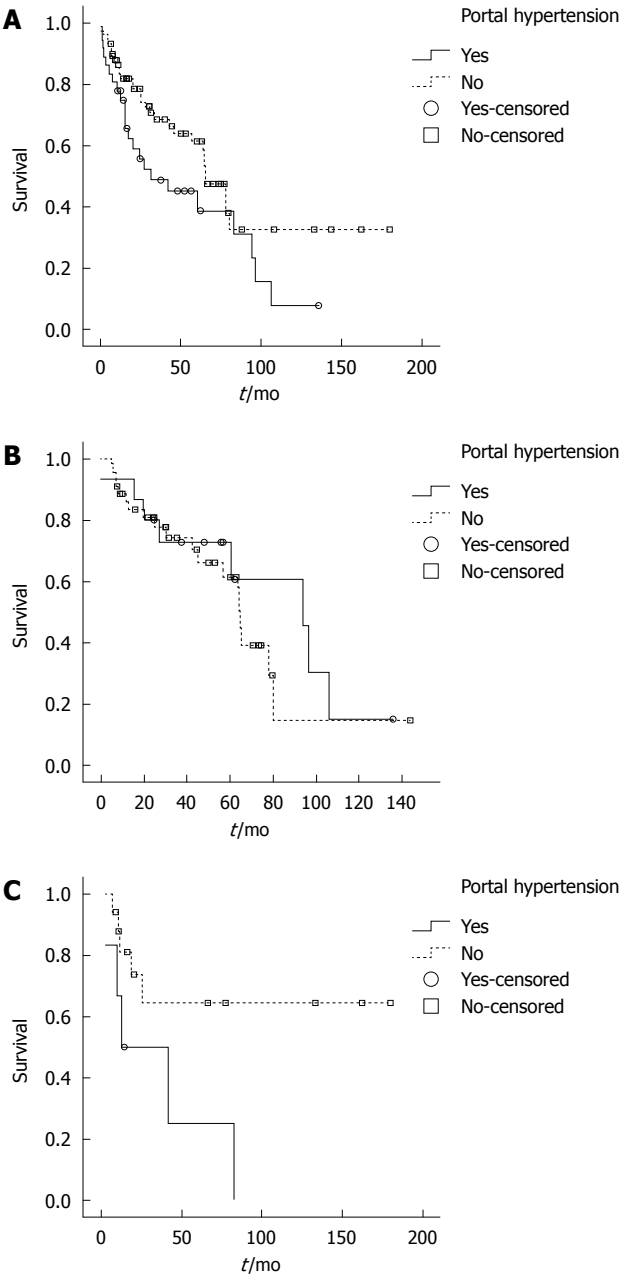

Figure 1 Overall survival analysis in patients. A: Overall survival analysis in patients with or without portal hypertension (PH); the difference between the two groups was statistically significant ($P = 0.04$); B: Overall survival analysis in Child-Pugh score (CP) A patients with or without PH, in the subgroup of patients submitted to limited resection ($P = 0.45$); C: Overall survival analysis in CP A patients with or without PH, in the subgroup of patients submitted to resection of 2 or more segments ($P = 0.03$).

on a group of 54 patients, in whom severity of PH was not worsened by liver resection. Llovet *et al*[29] in another study showed that clinical PH (assessed by the simultaneous presence of gastric-esophageal varices, thrombocytopenia lower than 100 000/mm^3 and splenomegaly, or on a portal-hepatic venous pressure gradient greater than 10 mm Hg) is a predictor of postoperative liver failure and that it is related to long term survival. These authors studied 77 patients divided into 3 groups based on the presence or absence of PH and bilirubin level. The 5-year survival of patients without PH was 74%, while the patients with PH and bilirubin lower than 1 mg/dL had a 5-year survival of 50%, and the patients with PH and bilirubin greater than 1 mg/dL had a 5-year survival of 25%. Other studies confirmed a correlation between PH and increased mortality and complications. Poon *et al*[30], in a study of 1222 patients undergoing liver resection for hepato-biliary cancers, showed that the presence of thrombocytopenia at the time of surgery is a risk factor in multivariate analysis for surgical complications. Similarly, Jarnagin *et al*[31] in a study of 1803 patients showed that preoperative thrombocytopenia is associated with a postoperative increased risk of mortality.

On the contrary, other authors did not detect a significant correlation between PH and liver failure after surgery[32,33]. Ishizawa *et al*[23] analyzed 322 Child-Pugh class A patients and found good long term results in patients with or without PH, (3- and 5-year survival of 71% and 56% *vs* 81% and 71% respectively, $P = 0.008$). Capussotti *et al*[24], in a study of 217 patients including 99 with PH at the time of surgery, showed that the 3- and 5-year survival rates are greater in patients without the presence of PH (62% and 40% *vs* 45% and 29% respectively, $P = 0.020$). However, resection in patients with PH and good hepatic function (Child-Pugh A) had similar results in terms of 3- and 5-year survival (65% and 41% *vs* 60% and 41%, $P = 0.503$). This study also shows that patients with PH have a higher incidence of postoperative complications, particularly those related to the deterioration of liver function (27% *vs* 15%, $P = 0.030$). Kawano *et al*[25] evaluated the results of liver resection in patients with esophageal varices. This study found that patients with esophageal varices had a better 5-year survival (70% *vs* 47%, $P = 0.045$); the authors underlined that patients with PH had a more frequent early diagnosis of HCC due to more careful follow up. More recently, Choi *et al*[34] reported that the 5-year survival rate of patients with clinical PH affected by single nodular HCC without macrovascular invasion was 78.4%, even if in Child-Pugh A cirrhotic patients, the presence of clinically significant PH was significantly associated with postoperative hepatic failure and poor prognosis after resection of HCC.

The data from our study confirm the recent experiences in the literature. Patients with PH at the time of surgery showed worse liver function and this justifies the increased number of complications related to the deterioration of liver function and the increased postoperative 3-mo mortality. Long term survival was significantly related to PH with significantly shorter survival ($P < 0.04$).

Among different Child-Pugh class patients we did not observe statistically significant differences in 3- and 5-year survival between patients with or without PH. In Child-Pugh class A patients submitted to minor resection, survival was not significantly affected by PH. Our study is the first in the literature to demonstrate the relationship between survival, PH and extent of hepatectomy. Surgery can produce good long term survival in patients with PH submitted to limited resection; conversely, PH had strong

adverse prognostic significance in patients who underwent resection of two or more segments. This data can help in the selection of patients and improve safety and long term results of surgery, as well as identify a group of patients who require resection of 2 or more segments, in whom resection is contraindicated and other non-surgical therapies should be applied.

This is a retrospective analysis from a single center. This allowed homogeneous data; however, multi-center studies are needed to confirm these results to reduce technical bias.

Our study confirms that the presence of PH at the time of surgery is not an absolute contraindication to resection in patients with liver cirrhosis. Although the rate of postoperative complications in patients with PH is greater, the results in terms of survival in the group of Child-Pugh class A patients is similar in patients without PH. Also, in patients with PH, limited liver resection can be performed with results comparable to those in patients without PH. Conversely, surgical resection of 2 or more segments in patients with PH results in significantly shorter survival and should not be recommended.

COMMENTS

Background

The presence of liver cirrhosis is the most important risk factor for the development of hepatocellular carcinoma (HCC): 85%-95% of HCCs arise in cirrhotic livers. Portal hypertension (PH) is related to increase in intrahepatic resistance due to the structural subversion of the cirrhotic liver and loss of vascular bed, and bleeding from gastro-esophageal varices is one of the most important complications of cirrhosis. The European Association for the Study of the Liver/American Association for the Study of Liver Diseases (AASLD) guidelines consider PH as a relative contraindication to liver resection, because of the high risk of postoperative liver failure, as reported in some clinical series. On the contrary, other authors have not detected a significant correlation between PH and liver failure after surgery.

Research frontiers

Surgical treatment is the most effective treatment for HCC and the mortality after surgery has decreased in recent years in relation to improved surgical techniques and peri-operative management of patients. Moreover, only about 30% of patients affected by HCC can be submitted to surgery, because of the characteristics of the tumor (stage, number of nodules, size, presence of vascular invasion), the general condition of the patient, or liver functional impairment, such as PH.

Innovations and breakthroughs

Recently, authors have not detected a significant correlation between PH and liver failure after surgery. The data from this study confirm the recent experiences in the literature. Survival analysis in patients with Child-Pugh A and B cirrhosis did not demonstrate significant differences in patients with or without PH. Moreover, limited resections (wedge or one segment) showed no statistical differences between patients with or without PH. When resection of two or more segments was performed, survival was significantly longer in patients without PH. Multivariate analysis with Cox's regression model confirmed that Child-Pugh, but not PH and type of hepatectomy class, was related to survival. This study is the first in the literature to demonstrate the relationship between survival, PH and extent of hepatectomy. Surgery can result in good long term survival in patients with PH submitted to limited resection; conversely, PH had strong adverse prognostic significance in patients who underwent resection of two or more segments.

Applications

This study confirms that the presence of PH at the time of surgery is not an absolute contraindication to resection in patients with liver cirrhosis. Results in terms of survival in the group of Child-Pugh class A patients are similar to patients without PH. Furthermore, in patients with PH, limited liver resection can be performed with results comparable to those in patients without PH. Conversely, surgical resection of 2 or more segments in patients with PH resulted in significantly shorter survival and is not to be recommended.

Terminology

Clinical PH is defined according to AASLD guidelines as the presence of esophageal varices or thrombocytopenia (platelet count < 100 000/mm^3), associated with splenomegaly.

Peer review

In this retrospective study, the authors compare the results of liver resection for HCC in patients with and without PH. The authors show that resection is safe in CTP. A patients regardless of PH, in patients undergoing limited resection. The authors show no difference in survival between those with and without PH in CTP A/B patients.

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BRIEF ARTICLE

Evaluation of latent links between irritable bowel syndrome and sleep quality

Massimo Bellini, Angelo Gemignani, Dario Gambaccini, Simona Toti, Danilo Menicucci, Cristina Stasi, Francesco Costa, Maria Gloria Mumolo, Angelo Ricchiuti, Remo Bedini, Nicola de Bortoli, Santino Marchi

Massimo Bellini, Dario Gambaccini, Cristina Stasi, Francesco Costa, Maria Gloria Mumolo, Angelo Ricchiuti, Nicola de Bortoli, Santino Marchi, Gastrointestinal Unit, Department of Gastroenterology, University of Pisa, 56100 Pisa, Italy
Angelo Gemignani, Danilo Menicucci, Remo Bedini, Institute of Clinical Physiology, National Research Council, 56100 Pisa, Italy
Angelo Gemignani, Danilo Menicucci, Remo Bedini, Extreme Centre, Scuola Superiore S Anna, 56100 Pisa, Italy
Angelo Gemignani, Department of Physiological Science, University of Pisa, 56100 Pisa, Italy
Simona Toti, Italian National Institute of Statistics, 00185 Rome, Italy
Author contributions: Bellini M contributed to patient enrollment, diagnosis of IBS, experimental design, and writing the manuscript; Gemignani A contributed to assessment of sleep disturbances, experimental design, and writing the manuscript; Gambaccini D contributed to patient enrollment, diagnosis of IBS, and writing the manuscript; Toti S and Menicucci D contributed to application of the IRT model and statistical analysis; Stasi C, Mumolo MG and de Bortoli N contributed to patient enrollment and diagnosis of IBS; Costa F performed lower digestive endoscopy in patients > 50 years old to exclude non-functional GI disorders; Ricchiuti A performed in all patients abdominal ultrasonography to exclude non-functional GI disorders; Bedini R contributed to statistical analysis and funded the study; Marchi S contributed to patient enrollment, diagnosis of IBS and funded the study.
Correspondence to: Dr. Massimo Bellini, Gastrointestinal Unit, Department of Gastroenterology, University of Pisa, Via Paradisa No. 2, 56100 Pisa, Italy. mbellini@med.unipi.it
Telephone: +39-50-997448 Fax: +39-50-997398
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Abstract

AIM: To examine the links between quality of sleep and the severity of intestinal symptoms in irritable bowel syndrome (IBS).

METHODS: One hundred and forty-two outpatients (110 female, 32 male) who met the Rome Ⅲ criteria for IBS with no psychiatric comorbidity were consecutively enrolled in this study. Data on age, body mass index (BMI), and a set of life-habit variables were recorded, and IBS symptoms and sleep quality were evaluated using the questionnaires IBS Symptom Severity Score (IBS-SSS) and Pittsburgh Sleep Quality Index (PSQI). The association between severity of IBS and sleep disturbances was evaluated by comparing the global IBS-SSS and PSQI score (Pearson's correlation and Fisher's exact test) and then analyzing the individual items of the IBS-SSS and PSQI questionnaires by a unitary bowel-sleep model based on item response theory (IRT).

RESULTS: IBS-SSS ranged from mild to severe (120-470). The global PSQI score ranged from 1 to 17 (median 5), and 60 patients were found to be poor sleepers (PSQI > 5). The correlation between the global IBS-SSS and PSQI score indicated a weak association (r = 0.2 and 95% CI: -0.03 to 0.35, $P < 0.05$), which becomes stronger using our unitary model. Indeed, the IBS and sleep disturbances severities, estimated as latent variables, resulted significantly high intra-subject correlation (posterior mean of r = 0.45 and 95% CI: 0.17 to 0.70, $P < 0.05$). Moreover, the correlations between patient features (age, sex, BMI, daily coffee and alcohol intake) and IBS and sleep disturbances were also analyzed through our unitary model. Age was a significant regressor, with patients ⩽ 50 years old showing more severe bowel disturbances (posterior mean = -0.38, $P < 0.05$) and less severe sleep disturbances (posterior mean = 0.49, $P < 0.05$) than older patients. Higher daily coffee intake was correlated with a lower severity of bowel disturbances (posterior mean = -0.31, $P < 0.05$). Sex (female) and daily alcohol intake (modest) were correlated with less severe sleep disturbances.

CONCLUSION: The unitary bowel-sleep model based on IRT revealed a strong positive correlation between the severity of IBS symptoms and sleep disturbances.

Key words: Irritable bowel syndrome; Sleep disorders; Item response theory model; Bayesian model

Peer reviewer: Guang-Yin Xu, MD, PhD, Assistant Professor, Division of Gastroenterology, Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX 77555-0655, United States

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INTRODUCTION

Irritable bowel syndrome (IBS) is quite prevalent in the general population (5%-20%) and is in fact the functional gastrointestinal disorder most frequently encountered in primary and secondary care[1,2]. IBS is characterized by abdominal discomfort, pain and changes in bowel habits (constipation and/or diarrhea)[3]. Visceral hypersensitivity, slowed gastrointestinal transit, and alterations in secretion activity are also often reported[4]. The direct and indirect costs of the syndrome are significant. IBS can have a serious impact on quality of life, and because it is often associated with other disorders, the patient may have to undergo expensive tests and treatments[5-7].

Sleep disturbances connected with gastrointestinal functional disorders, in particular IBS, have been reported[8-10], but no consistent abnormality in sleep patterns has been identified, apart from a significant increase in rapid-eye-movement (REM) sleep[11,12]. Many studies have associated sleep alterations with severity of IBS symptoms[12], but it is not yet known whether there is a direct pathophysiological relationship[13,14]. To date, a fundamental question remains - is sleep disturbance a cause or a consequence of IBS[15]?

Growing awareness of the importance of the "brain-gut axis" has led some gastroenterologists to investigate sleep as an independent factor in the pathogenesis and clinical expression of IBS[11,16].

The aim of the present study was to evaluate the association between the severity of sleep disturbances and intestinal symptoms, measured using two widely accepted questionnaires: the IBS Symptom Severity Score (IBS-SSS)[17] and the Pittsburgh Sleep Quality Index (PSQI)[18]. In addition, individual patient features were also analyzed to investigate their possible interaction with the severity of bowel and sleep disturbances and to exclude their potential confounding role.

MATERIALS AND METHODS

Study patients

Between October 2007 and September 2008, 142 patients who met the Rome Ⅲ criteria for IBS[3] with no confounding psychiatric comorbidity (diagnosed according to DSM Ⅳ axis I criteria) were consecutively enrolled from outpatients attending the Gastrointestinal Unit of the University of Pisa. All patients gave their informed written consent as required by the University Ethics Committee for Clinical Studies. The cohort was comprised of 110 female and 32 male patients (median age: 38 years; range: 18-79 years), of whom, 52 had constipation as the predominant symptom (C-IBS), 48 had diarrhea as the predominant symptom (D-IBS), and 42 had mixed symptoms (M-IBS).

We also evaluated physical and life-habit variables in each patient (Table 1): (1) sex and age; (2) body mass index (BMI)[19]; (3) smoking; (4) alcohol intake[20]; (5) coffee intake; (6) physical activity; and (7) water intake.

Table 1 Clinical subtypes, physical and life-habit variables of irritable bowel syndrome patients

	Item categories	Patients (%)
IBS subtype	M-IBS	29.6
	D-IBS	33.8
	C-IBS	36.6
Age (yr)	≤ 50	78.9
	> 50	21.1
Sex	Male	22.5
	Female	77.5
BMI (kg/m^2)	≤ 25	76.1
	> 25	23.9
Smoking	No	74.6
	Yes	25.4
Alcohol consumption	Non-/occasional drinkers	82.4
	Light drinkers (< 30 g ethanol/d)	17.6
Coffee consumption	< 2 cups/d	55.6
	≥ 2 cups/d	44.4
Physical activity	No	66.2
	Yes	33.8
Water consumption	< 1.5 L/d	61.3
	≥ 1.5 L/d	38.7

IBS: Irritable bowel syndrome; BMI: Body mass index.

IBS and sleep questionnaires

IBS symptoms were evaluated using the IBS-SSS questionnaire[17], which measured five separate items (Table 2): (1) presence and severity of abdominal pain or discomfort; (2) frequency of abdominal pain or discomfort; (3) presence and severity of abdominal distension; (4) degree of satisfaction with defecatory function; and (5) degree of interference of IBS symptoms with daily lifestyle. For each of the five items, the questions generated a score ranging from 0 to 100 that, for the purposes of analysis, was divided into three consecutive intervals: low, medium, and high (Table 2). The scores for the five items were summed to arrive at a global IBS-SSS (range: 0-500) and the IBS severity was then classified as mild (75-175), moderate (175-300) or severe (≥ 300).

Sleep quality was evaluated using the PSQI[18], a self-administered questionnaire based on seven items: subjective sleep quality, sleep latency, sleep duration, habitual

Table 2 Distribution of responses to the items in the irritable bowel syndrome Symptom Severity Score across categories (low, medium and high)

IBS-SSS items	Category (score interval)	Patients (%)
Pain severity	Low: ≤ 40	31.0
	Medium: 40-70	43.7
	High: > 70	25.4
Pain frequency	Low: ≤ 20	19.7
	Medium: 20-70	33.8
	High: > 70	46.5
Distension	Low: ≤ 40	30.3
	Medium: 40-70	28.2
	High: > 70	41.5
Dissatisfaction with defecatory function	Low: ≤ 40	12.7
	Medium: 40-70	26.8
	High: > 70	60.6
Interference with daily life style	Low: ≤ 40	28.9
	Medium: 40-70	32.4
	High: > 70	38.7

IBS-SSS: Irritable bowel syndrome Symptom Severity Score.

sleep efficiency, sleep disturbances, use of sleep inducers, and daytime dysfunction. Each item was scored from 0 to 3, and the PSQI global index was calculated by summing these scores (range: 0-21); a global score > 5 identified poor sleepers.

In both questionnaires, lower values indicated lower severity, and higher values a more severe condition.

Statistical analysis

The association between the severity of IBS symptoms and sleep disturbances was first verified by directly comparing the global IBS-SSS and PSQI score, and then by evaluating the links between individual items in the two questionnaires.

For the first approach, we used both the Pearson correlation coefficient and the generalized Fisher exact test[21]. The Pearson correlation measured the linear dependence between global scores, whereas the Fisher exact test verified the association between IBS-SSS and PSQI through a contingency table.

We adopted a unitary bowel-sleep model based on item response theory (IRT) that enabled us to investigate directly the dependences between IBS-SSS and PSQI single items. Furthermore, we also evaluated the weight of patient features and life habits in affecting the severity of IBS and sleep disturbances.

By focusing on individual items as the unit of analysis rather than the global score, the IRT model could circumvent the problem of different patterns of responses generating identical global scores. To this end, the values of both IBS-SSS single items and patient features were transformed in categories as indicated in the Tables 1 and 2. At variance, the PSQI items were not transformed since each item could have only four values (0-3), thus PSQI single item categories corresponded to the item values.

The output of our IRT model was the estimate of two latent variables: one related to the severity of IBS, and the other to sleep disturbances. In order to evaluate the possible intra-subject associations between IBS and sleep disturbances, the latent variables for the two were jointly modeled using a bivariate normal distribution. The strength of this association was quantified by the covariance parameter. Specifically, we adopted the partial credit model[22], which could be used for the polytomous ordered categories for each item. The partial credit model postulated that the probability of a given response with respect to the next response was a function (logistic curve) of the severity of the subject's symptoms and of structural parameters. The severity of symptoms, considered as a latent variable, was assumed to have a normal distribution[23]. Finally, we used a regression model to remove the effects of the patient features from the latent severities, and to identify the features with a significant effect on each latent severity variable.

In the partial credit model analysis, a sum-to-zero constraint was imposed on the structural parameters[24]. The model was estimated within a Bayesian framework by means of a Markov-chain Monte Carlo algorithm with Gibbs sampling (using WinBUGS 1.4)[25]. This estimation required that one specified the prior distributions of all parameters, but in the present study, no such a priori information was available; thus non-informative priors (i.e., parameter-flat a priori distributions) were used.

The Bayesian method enabled us to estimate the distribution of each parameter (posterior distribution), from which we could derive the central tendency (posterior mean) along with the Bayesian confidence (or Credibility) interval (CI).

In summary, the unitary bowel-sleep model yielded estimates for each patient of the latent severity of their IBS and sleep disturbances, with their covariance parameters.

Finally, the mean latent severity of IBS and sleep disturbances were correlated with patient features (including IBS subtypes). One category was maintained as the reference and we verified whether the regression coefficient (posterior mean), describing the change from the reference to every other category, was significantly different from zero.

RESULTS

Analysis of the global scores

Patients had IBS-SSS ranging from 120 to 470 (median 280): 10 subjects were classified as mild, 69 as moderate, and 63 as severe. In Table 2, the distribution of responses to the items in the IBS-SSS across categories (low, medium and high) is shown.

The sleep quality in our patients ranged from 1 to 17, with a median PSQI score of 5. Sixty patients were poor sleepers (global PSQI > 5). The distribution of the scores for the items in the PSQI is shown in Table 3.

The correlation between the severity of IBS and sleep disturbances was evaluated by comparing the global IBS-SSS and PSQI scores. Pearson's *r* was found to be 0.2

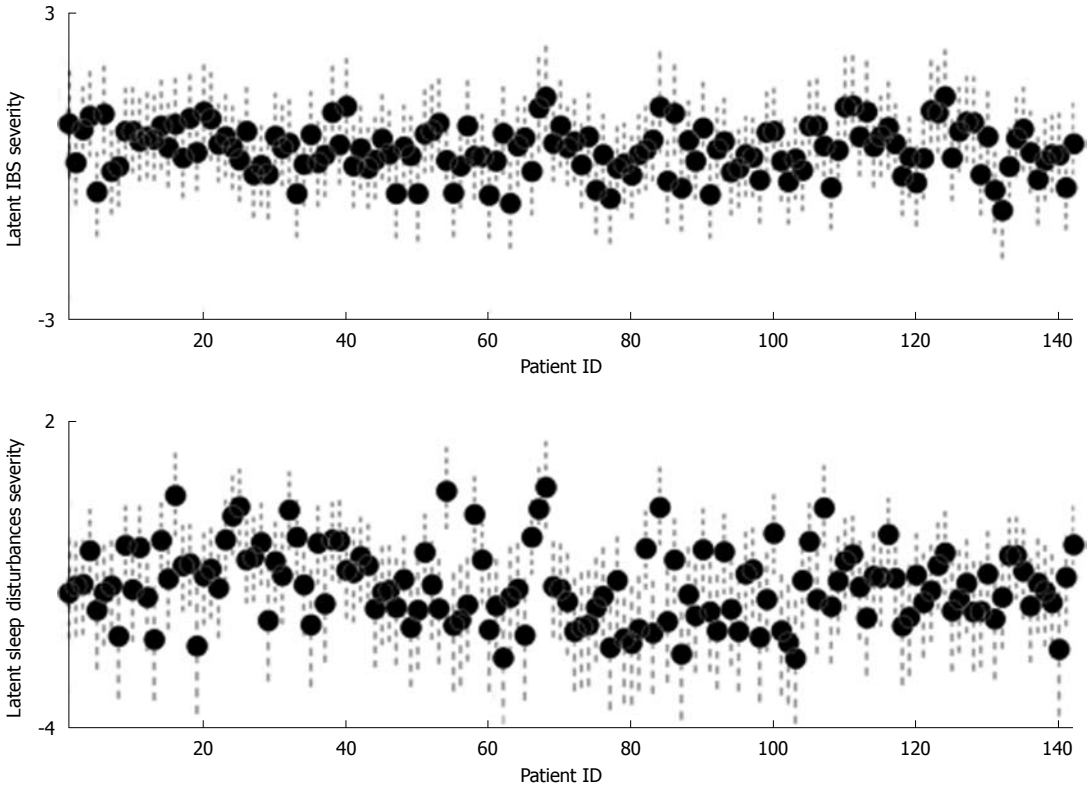

Figure 1 Irritable bowel syndrome latent severity in each patient, with 95% CI (top) and sleep disturbances latent severity in each patient, with 95% CI (bottom). Error bars indicate CI. ID indicates the label of each patient. IBS: Irritable bowel syndrome. CI: Confidence Interval.

Table 3 Distribution of responses to Pittsburgh Sleep Quality Index items

PSQI items	Item scores	Patients (%)
Subjective sleep quality	0	14.1
	1	62.7
	2	20.4
	3	2.8
Sleep latency	0	52.8
	1	28.2
	2	14.1
	3	4.9
Sleep duration	0	43.7
	1	35.9
	2	14.8
	3	5.6
Habitual sleep efficiency	0	54.9
	1	33.8
	2	7.7
	3	3.5
Sleep disturbances	0	8.5
	1	81.0
	2	9.9
	3	0.7
Use of sleeping medication	0	81.0
	1	3.5
	2	2.1
	3	13.4
Daytime dysfunction	0	31.0
	1	50.7
	2	13.4
	3	4.9

PSQI: Pittsburgh Sleep Quality Index.

Table 4 Contingency table for the global irritable bowel syndrome Symptom Severity Score and global Pittsburgh Sleep Quality Index score

	Global PSQI score	
	Good	Poor
Global IBS-SSS		
Mild	7	3
Moderate	44	25
Severe	31	32

IBS-SSS: Irritable bowel syndrome Symptom Severity Score; PSQI: Pittsburgh Sleep Quality Index.

(95% CI: 0.03 to 0.35), indicating a weak significant linear relationship between the two scores. Analysis of a contingency table for IBS-SSS *vs* PSQI categories using Fisher's exact test failed to detect any association between the two indices ($P = 0.18$) (Table 4).

Analysis of the latent severities

To complete the study of the questionnaires, the analysis was extended by modeling the responses to each single item with the partial credit models, and the regression analysis to determine the effect of the individual features on the estimated severity of IBS and sleep disturbances.

In Figure 1, the posterior means of the severity of IBS and sleep disturbances (expressed as latent variables) for the 142 patients are plotted (with their 95% CIs). The

Table 5 For each patient, feature regression coefficients (posterior means) and 95% confidence interval for the latent severities of both irritable bowel syndrome and sleep disturbances are shown

Patient features	Categories	IBS latent severity, coef (95% CI)	Sleep disturbances latent severity, coef (95% CI)
IBS subtype	Mixed	0	0
	Diarrhoea	-0.10 (-0.45 to 0.24)	0.02 (-0.42 to 0.46)
	Constipation	0.32 (-0.05 to 0.69)	0.16 (-0.30 to 0.65)
Age (yr)	≤ 50	0	0
	> 50	-0.38 (-0.75 to -0.02)[1]	0.49 (0.02 to 0.96)[1]
Sex	Male	0	0
	Female	0.10 (-0.24 to 0.46)	-0.53 (-0.95 to -0.07)[1]
BMI (kg/m^2)	≤ 25	0	0
	> 25	0.04 (-0.32 to 0.38)	0.09 (-0.33 to 0.52)
Smoking	No	0	0
	Yes	0.07 (-0.27 to 0.41)	-0.04 (0.45 to 0.38)
Alcohol consumption	Non-/occasional drinkers	0	0
	Light drinkers	-0.22 (-0.60 to 0.16)	-0.60 (-1.08 to -0.12)[1]
Coffee consumption	< 2 cups/d	0	0
	≥ 2 cups/d	-0.31 (-0.60 to -0.01)[1]	-0.08 (-0.44 to 0.26)
Physical activity	No	0	0
	Yes	-0.18 (-0.48 to 0.11)	-0.29 (-0.68 to 0.08)
Water consumption	< 1.5 L/d	0	0
	≥ 1.5 L/d	0.27 (-0.03 to 0.56)	0.07 (-0.29 to 0.42)

[1]Significant differences between covariables. IBS: Irritable bowel syndrome. CI: Confidence interval; BMI: Body mass index.

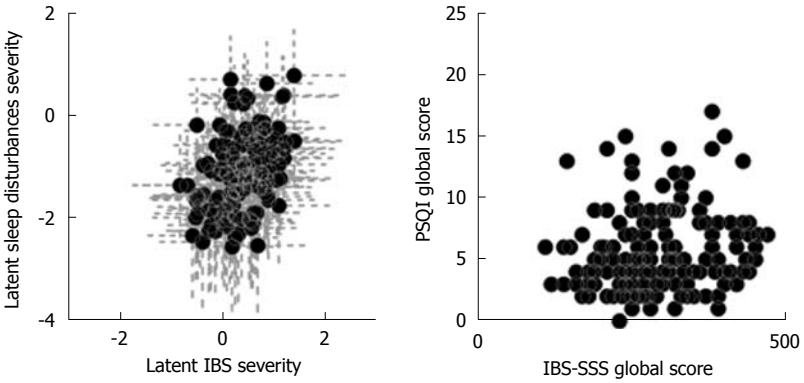

Figure 2 Scatter plot of the global Pittsburgh Sleep Quality Index score *vs* the global irritable bowel syndrome-Symptom Severity Score (left panel), and of sleep disturbances and irritable bowel syndrome latent severities (dots) (right panel). The grey lines indicate the 95% CI. CI: Confidence interval.

latent severity of sleep disturbances was a better discriminant between subjects than IBS, since it showed a more marked between-subject variability: all of the patients had a priori IBS symptoms, whereas the quality of their sleep was measured for the first time in this study.

Our unitary bowel-sleep model demonstrated a significant association between the severity of bowel symptoms and sleep disturbances. In contrast to the results of the global score comparison, a significant intra-subject correlation was found (posterior mean $r = 0.45$ and 95% CI: 0.17 to 0.70).

Figure 2 shows two scatter plots, on the left the IBS-SSS and PSQI global scores, whereas on the right, the two latent severities. The usage of the unitary bowel-sleep model provided a relationship between latent sleep disturbances severity and latent IBS severity. At variance, the usage of global scores did not provide any information about sleep and bowel dysfunction interactions. In the right panel of Figure 2, each black dot corresponds to a single patient and the dashed grey cross indicates the respective CI. Despite the variability between patients, the correlation between the two latent severities was evident, while the scatter plot on the left panel did not confirm any association between the global scores.

Furthermore, the unitary bowel-sleep model allowed us to identify a significant regression between IBS and sleep disturbances latent severities and patient features (Table 5).

The significance of a feature effect corresponded to a significant regression coefficient, and the sign of this coefficient indicated the direction of the relationship: a positive sign signified that the change of category (from the reference one to the other) came with a severity increase and *vice versa*; the 95% CI indicated the significance of the coefficient.

Among the individual features, age was a significant

regressor for both latent severities; young patients (≤ 50 years old) showed more severe bowel disturbances (posterior mean = -0.38) and less severe sleep disturbances (posterior mean = 0.49) than older patients. Daily coffee intake was significantly correlated with severity of bowel disturbances (posterior mean = -0.31); subjects who consumed ≥ 2 cups per day showed less severe bowel disturbances. Sex and daily alcohol intake were found to be significantly correlated with severity of sleep disturbances; female patients exhibited less severe sleep disturbances than male patients, while non-drinkers showed more severe sleep disturbances than those with a moderate alcohol intake. No other life-habit variables were found to be significantly correlated with latent severity of IBS or sleep disturbances.

DISCUSSION

The aim of this study was to evaluate the mutual influences between sleep disturbances and IBS in a large sample of IBS patients. The study provides a detailed statistical analysis of the possible association between bowel disorders, sleep disturbances and life habits.

Our sample may be considered as representative of the IBS population in Italy (this was confirmed by the distribution of IBS subtypes, age and BMI in the cohort). Therefore, our results can be compared and discussed in the light of the results of other studies on IBS patients.

Despite the fact that 42% of the participants had a global PSQI score > 5, only a weak and ambiguous association between IBS-SSS categories and PSQI categories was evident by using standard statistical analysis. Indeed, the correlation coefficient between the global scores was weakly significant and the Fischer exact test did not yield any association.

Conversely a strong link was highlighted by applying the IRT model[26,27] and linear regression.

IRT provides a framework to detect the effect of a series of variables (such as the responses to items on psychological tests) on latent traits of interest (IBS and sleep disturbances). In standard data analyses, the patient's raw score is calculated by summing the scores for the different items, without taking into account differences in the responses to specific items by different patients. It may arise that two patients have the same raw global score but different patterns of symptoms, or different raw global scores but similar symptom patterns. The inability to discriminate between the two possibilities can be resolved by using IRT model, which focuses on individual items rather than the global score. In addition, IRT allows one to introduce covariates (in our case age, BMI, and life habits) and analyze their effect on the parameters of interest.

The IRT model identified a latent link between sleep disturbances and IBS symptoms. These results showed that IBS patients suffered from a considerable degree of sleep impairment, and are in line with those of other studies[8,28,29]. In particular, a recent survey found that sleep disturbances were an independent predictor of IBS in nurses[8].

Through IRT analysis, we also determined that older age, male sex, and no alcohol intake were significant predictors of more severe sleep disturbances.

Our study found that female IBS patients complained with fewer sleep disturbances than males. This would appear to be inconsistent with studies in the general population, which show that females are more likely to suffer from sleep disorders[30], more susceptible to the effects of stress on sleep[31], and after menopause, run a higher risk of developing insomnia[30]. The present findings could be explained by the demographics of our sample, which were characterized by a relatively low mean age and hence a higher percentage of fertile women; indeed, IBS patients tend to be relatively young (< 50 years old)[3].

Evaluation of the predictive parameters for the latent severity of sleep disorders showed that age was positively correlated with sleep disturbances. These data come as no surprise, because aging is widely considered to be a triggering factor for insomnia[32-34].

Finally, this study showed that light alcohol intake was correlated with a lower latent severity of sleep disturbances. It has long been known that a little amount of alcohol consumed by healthy individuals before going to sleep shortens sleep latency, reduces REM sleep, and increases non-REM sleep[35-39]. Alcohol is frequently used as a form of self-medication by patients suffering from sleep disorders, especially insomnia[40].

More unexpectedly, we found that younger age and higher coffee intake were associated with less severe IBS symptoms. The first-line treatment for IBS usually consists of a change in lifestyle and diet, and drinking less coffee is often recommended, even though there are no studies demonstrating a link between the consumption of coffee and the severity of IBS symptoms. Sloots *et al*[41] have reported that the intake of 280 mL of coffee caused no change in rectal compliance or visceral sensibility. However, in our study, coffee drinkers actually consumed no more than two cups per day.

In conclusion, using IRT analysis we found that: (1) there was a positive association between the severity of IBS symptoms and sleep disturbances, and (2) some patient features were significant predictors of severity of IBS and sleep disturbances. These results are consistent with those of other studies[10,14]. The pathophysiological mechanism underlying this association is only partially understood, however. One possibility is that sleep disorders can induce visceral hyperalgesia, which increases the patient perception of gastrointestinal symptoms[8,15]. Several other factors could play a role, including endophenotypic traits such as those associated with stress susceptibility[42].

Further research is needed to clarify whether the association of IBS and sleep disturbances represents comorbidity or the expression of a single disturbance in the brain-gut axis.

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COMMENTS

Background

Sleep disturbances connected with gastrointestinal functional disorders, in particular irritable bowel syndrome (IBS), have been reported and some studies have associated sleep alterations with the severity of IBS symptoms. It is not yet known whether it is a simple comorbidity or if there is a direct pathophysiological relationship. To date, a fundamental question has arisen: is sleep disturbance a cause or a consequence of IBS?

Research frontiers

Sleep disturbances represent a negative factor affecting the outcome of several medical and psychiatric conditions, therefore, growing awareness has led some gastroenterologists to investigate sleep as an independent factor also in the pathogenesis and clinical expression of IBS.

Innovations and breakthroughs

Item response theory (IRT) for the first time has been used to identify latent links between digestive symptoms and sleep quality. Furthermore, this unitary bowel-sleep model enabled us to evaluate better the weight of patient features and life habits in the severity of IBS symptoms and sleep disturbances.

Applications

The results of this study suggest: (1) the use of IRT models for uncovering latent links in clinical comorbidity; (2) the importance of studying sleep quality in functional digestive disorders; and (3) further surveys on the clinical mutual interaction between sleep disturbances and functional digestive disorders (i.e., if the treatment of sleep disturbances improves digestive symptoms and/or *vice versa*)

Terminology

IRT is a paradigm for the analysis of tests and questionnaires. IRT models are often referred to as "latent trait models". The term latent is used to emphasize discrete item responses not directly observed but inferred from the manifest responses. IRT brings greater flexibility and provides more sophisticated information and is generally considered an improvement of classical test theory. The Bayesian method enables one to estimate the distribution of each parameter from which one could derive the central tendency.

Peer review

The authors have looked at sleep quality in patients with IBS to evaluate the relationship between digestive symptoms and sleep quality by using IRT analysis. The results are analyzed in detail and the statistical tool of IRT is used to obtain significant associations. The data are solid, the paper is interesting, has clinical significance and is well written.

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BRIEF ARTICLE

Serum leptin and ghrelin in chronic hepatitis C patients with steatosis

Christos Pavlidis, Georgios I Panoutsopoulos, Dina Tiniakos, Sotirios Koutsounas, John Vlachogiannakos, Irini Zouboulis-Vafiadis

Christos Pavlidis, John Vlachogiannakos, Irini Zouboulis-Vafiadis, First Department of Propaedeutic Medicine "LAIKO" General Hospital, Agiou Thoma 17, Athens 11527, Greece
Georgios I Panoutsopoulos, Department of Nursing, University of Peloponnese, Orthias Artemidos and Plateon, Sparta, Lakonia 23100, Greece
Dina Tiniakos, Laboratory of Histology and Embryology, Medical School, National and Kapodistrian University of Athens, Mikras Asias 75, Athens 11527, Greece
Sotirios Koutsounas, Reference Center for Viral Hepatitis, IKA, Athens 10436, Greece
Author contributions: Pavlidis C and Panoutsopoulos GI contributed equally to this work; Pavlidis C and Zoumboulis-Vafiadis I designed the study; Pavlidis C, Zoumboulis-Vafiadis I, Tiniakos D and Koutsounas S performed the study; Panoutsopoulos GI analyzed the data; Pavlidis C, Panoutsopoulos GI, Zoumboulis-Vafiadis I, Tiniakos D and Vlachogiannakos J wrote the manuscript.
Correspondence to: Dr. Christos Pavlidis, First Department of Propaedeutic Medicine "LAIKO" General Hospital, Agiou Thoma 17, Athens 11527, Greece. chrispavlidis@hotmail.com
Telephone: +30-210-6002252 Fax: +30-266-1046106
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Abstract

AIM: To determine the associations between leptin and ghrelin concentrations and sustained virological response (SVR) in chronic hepatitis C patients with steatosis.

METHODS: We retrospectively assessed 56 patients infected with hepatitis C virus (HCV) genotype-1 and 40 with HCV genotype-3. Patients with decompensated cirrhosis, and those with other causes of chronic liver disease, were excluded. Serum HCV-RNA concentrations were measured before the initiation of treatment; at weeks 12 (for genotype 1 patients), 24 and 48 during treatment; and 24 wk after the end of treatment. Genotype was determined using INNO-LIPA HCV assays, and serum leptin and ghrelin concentrations were measured using enzyme-linked immunosorbent assay. Biopsy specimens were scored according to the Ishak system and steatosis was graded as mild, moderate, or severe, according to the Brunt classification.

RESULTS: Overall, SVR was positively related to the presence of genotype-3, to biopsy-determined lower histological stage of liver disease, and lower grade of steatosis. Patients $\geqslant$ 40 years old tended to be less responsive to therapy. In genotype-1 infected patients, SVR was associated with a lower grade of liver steatosis, milder fibrosis, and an absence of insulin resistance. Genotype-1 infected patients who did not achieve SVR had significantly higher leptin concentrations at baseline, with significant increases as the severity of steatosis worsened, whereas those who achieved SVR had higher ghrelin concentrations. In genotype-3 infected patients, SVR was associated only with fibrosis stage and lower homeostasis model assessment insulin resistance at baseline, but not with the degree of steatosis or leptin concentrations. Genotype-3 infected patients who achieved SVR showed significant decreases in ghrelin concentration at end of treatment. Baseline ghrelin concentrations were elevated in responders of both genotypes who had moderate and severe steatosis.

CONCLUSION: Increased serum leptin before treatment may predict non-SVR, especially in HCV genotype-1 infected patients, whereas increased ghrelin may predict SVR in genotype-1.

Key words: Hepatitis C virus; Steatosis; Leptin; Ghrelin; Sustained virological response

Peer reviewer: Rashmi, Kaul, PhD, Assistant Professor, Department of Biochemistry and Microbiology, Oklahoma State

University-Center for Health Sciences, 1111 W 17th St, Tulsa, OK 74107, United States

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INTRODUCTION

Hepatic steatosis is a histopathological feature observed in > 50% of patients with chronic hepatitis C (CHC)[1,2], but occurs less frequently in patients with chronic hepatitis B (27%-51%) and autoimmune hepatitis (16%-19%)[3,4]. Steatosis in CHC patients has been attributed to a combination of mechanisms involved in the pathogenesis of non-alcoholic fatty liver disease, as well as to the direct effect of hepatitis C virus (HCV) on hepatic lipid metabolism leading to triglyceride accumulation[5,6]. In contrast, steatosis in patients chronically infected with hepatitis B virus (HBV) is associated with host metabolic factors[7].

Leptin is an adipokine that contributes to the pathogenesis of liver steatosis[8,9]. In patients with CHC, higher serum leptin concentrations have been associated with the presence of steatosis[10]. Although no clear correlation has been observed between leptin concentrations and the extent of steatosis[11], a recent study reported that high serum leptin concentrations correlated with more severe steatosis, lower viremia, and a lower antiviral response, mainly in patients infected with HCV genotype-1, which constituted 71% of the study population[12].

Leptin, the product of the obese (ob) gene, is mainly expressed by adipose tissue, although it is expressed in other organs, including the liver[13]. Leptin plays an important role in the regulation and metabolism of body fat and may induce insulin resistance, increase fatty acid concentrations in the liver, and enhance lipid peroxidation[5,8,9]. Leptin may act as an immunomodulator, inducing the release of cytokines, such as tumor necrosis factor (TNF)-α, interferon (INF)-γ, interleukin (IL)-18, and tumor growth factor (TGF)-β1, thus promoting liver steatosis and fibrosis[8].

Ghrelin is a peptide that acts as an endogenous ligand of the growth hormone secretatog receptor[14]. Ghrelin is involved in energy metabolism, food intake, and glucose homeostasis[14,15]. Recent studies have assessed whether ghrelin acts as an independent signal of adiposity or as a downstream mediator of leptin, affecting energy balance[16].

Little is known about serum ghrelin concentrations in patients with CHC and steatosis, or on the effects of ghrelin concentration on treatment response. We therefore assessed whether pretreatment serum leptin and ghrelin concentrations differ in steatotic patients infected with HCV genotypes-1 and -3, and whether these concentrations are associated with response to antiviral treatment. We also evaluated the correlations between pretreatment serum leptin and ghrelin concentrations and liver histology and metabolic factors, as well as determining whether the impact of antiviral treatment on leptin and ghrelin concentrations differed by HCV genotype.

MATERIALS AND METHODS

Patient population

We retrospectively assessed patients with serologically, virologically, and histologically confirmed CHC, recruited between 2005 and 2008. Patients were included if they had detectable anti-HCV antibody by enzyme-linked immunosorbent assay (ELISA) Ⅲ at least twice a year, detectable serum HCV-RNA by a sensitive PCR assay within 1 mo prior to the start of treatment, liver biopsy showing chronic hepatitis with steatosis within 6 mo before treatment, and elevated alanine aminotransferase (ALT) activity (> 40 IU/L and < 400 IU/L) at entry and at least once during the 6 mo before the first screening.

Patients with decompensated cirrhosis; other causes of chronic liver disease; a history of intravenous drug abuse or alcohol consumption; use of hepatotoxic drugs, herbal medications or immunosuppressive agents; diabetes; thyroid disorders; chronic renal failure; serious psychiatric disorders; HIV or HBV co-infection; or hepatocellular carcinoma, were excluded.

None of these patients had previously received antiviral treatment or steatosis-inducing therapy. The duration of HCV treatment with PEGylated INF-α and ribavirin was genotype-based. Genotype 1 patients who did not achieve undetectable HCV-RNA or a decrease in 2 logs of HCV-RNA at week 12 (early virological response or EVR), were considered non-responders but included in the study. All patients were clinically, hematologically and biochemically evaluated at weeks 2, 4, 8, 12, 24, 48, and 72 after the start of treatment.

The study protocol was approved by our institutional review board, and all patients provided written informed consent. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Body composition measurements

Height and weight were determined at baseline, and body mass index (BMI), calculated as weight (in kg)/height (in m)^2, was determined at baseline and at the end of treatment. Waist circumference indicative of visceral obesity was defined as > 102 cm in men and > 88 cm in women.

Laboratory and virology assays

Blood samples were obtained from all subjects after overnight fasting, and serum was obtained after centrifugation and stored in aliquots at -70℃ until assayed. Routine biochemical (aspartate aminotransferase, ALT, γ-glutamyl transpeptidase, alkaline phosphatase, glucose, urea, creatinine, cholesterol, triglycerides, albumin) and

hematological (hemoglobin, white blood cells, platelets) were performed using automated techniques.

Serological markers (hepatitis B surface antigen, anti-HBs, hepatitis B e antigen, anti-HBe, anti-HBc total, anti-HCV, anti-HDV, anti-HIV1,2, and anti-HAV IgM and IgG) were assayed using commercially available enzyme immunoassays (Abbott Laboratories, United States). Serum HCV-RNA levels were measured with a PCR assay (Cobas Amplicor HCV version 2, Roche Diagnostics, United States), both qualitatively (lower detection limit of 50 IU/mL) and quantitatively (range of detection 600-10^6 IU/mL). HCV genotype was determined using an INNO-LIPA HCV assay (Innogenetics, Belgium). Serum HCV-RNA concentrations were measured before the initiation of treatment, at weeks 12 (for genotype 1 patients), 24, and 48 during treatment, and 24 wk after the end of treatment. Serum leptin and ghrelin concentrations were measured using ELISA kits (BioVendor Laboratory, United States), at screening and at the end of treatment and expressed as ng/mL. Insulin resistance index was calculated as: insulin resistance [homeostasis model assessment insulin resistance (HOMA-IR)] = fasting insulin (mIU/L) × fasting glucose (mmol/L)/22.5.

Liver histology

Liver biopsies were obtained at baseline using the Menghini technique (mean length of biopsy cores, 1.7 cm). Liver tissue was fixed in 10% neutral formalin and paraffin-embedded sections were stained with hematoxylin-eosin and Masson trichrome stains. Liver biopsies were scored by an experienced liver pathologist using the Ishak scoring system. Steatosis was quantified as the percentage of hepatocytes that contained fat droplets and was graded using a three-tier scale: grade 1/mild (1%-33%), 2/moderate (33%-66%), and 3/severe (> 66%). Patients with histopathological findings of steatohepatitis, including hepatocellular ballooning or perisinusoidal fibrosis in zone 3, were excluded.

Statistical analysis

All continuous variables are presented as mean ± SD or medians ± interquartile ranges (75th-25th percentiles) if they deviated from normality. The association between each genotype and continuous variables was determined by ANOVA (using post-hoc Scheffe's test when the omnibus test was significant) or Student's *t*-test. The association between each genotype and categorical variables was determined using the χ^2 test. Multiple logistic regression analyses were used to determine the association between response to therapy and leptin concentrations, ghrelin concentrations, hepatic steatosis, fibrosis, and HOMA-IR, with results presented as odds ratios and 95% CI. These analyses were performed separately for each genotype and for measurements at baseline and at the end of follow up. For reasons of multicollinearity, it was not possible to include steatosis and fibrosis in the same model. Therefore, fibrosis and steatosis were alternatively introduced (one at a time) into the core model. Mixed effect models were used to examine the relationship between baseline and end of follow-up leptin and ghrelin concentrations among responders and non-responders. There were no significant changes in weight or fat composition in the study population during treatment; therefore, there was no need to adjust leptin and ghrelin concentrations for BMI after treatment. All reported probability values (*P*-values) were based on two-sided tests, with significance set at 0.05. All statistical analyses were performed using the SAS statistical package (Version 9.1, SAS Institute Inc., NC).

Table 1 Clinical, virological, and histological data of the study population (mean ± SD) *n* (%)

	SVR (*n* = 62)	Non-SVR (*n* = 34)	*P*-value
Age	35.01 ± 1.57	39.21 ± 2.07	0.050
Sex (male/female)	36/26	21/13	
BMI	24.700 ± 0.179	25.530 ± 0.637	0.060
Genotype-1	32 (57.1)	24 (42.9)	
Genotype-3	30 (75)	10 (25)	
Stage (0-6)	1.661 ± 0.095	2.412 ± 0.141	< 0.001
Grade (0-18)	5.371 ± 0.136	5.265 ± 0.204	NS
Steatosis (1-3)	1.565 ± 0.082	2.060 ± 0.126	0.001

NS: Non-significant; SVR: Sustained virological response; BMI: Body mass index.

RESULTS

Of the 154 treatment-naive patients with CHC screened, 96 fulfilled our enrollment criteria and completed treatment. The study population consisted of 60 men and 36 women, all of Caucasian origin; of these, 56 were infected with HCV genotype-1 and 40 with HCV genotype-3.

HCV genotypes and response to treatment

All patients received combination therapy with PEGylated INF a-2b or a-2a, plus weight-adjusted ribavirin, for 24 (genotype-3) or 48 (genotype-1) weeks. All tolerated treatment well and completed treatment without any major side effects, significant reductions in drug dose, and significant changes in BMI (reduction more than 2 kg/m^2). Patients infected with HCV genotype-1 who did not achieve sustained virological response (SVR) stopped therapy, whereas those infected with HCV genotype-3 were treated for 24 wk without measurements of HCV-RNA at week 12, according to currently approved treatment guidelines. Of the 96 patients, 62 (64.6%) achieved SVR, whereas the remaining 34 (35.4%) did not.

Clinical, virological, and histological data of patients of both genotypes who did and did not attain SVR are presented in Table 1. Of the 56 patients with genotype 1, 32 (57.1%) achieved SVR, compared with 30 of 40 patients (75%) with genotype-3; thus SVR was significantly related to infection with genotype-3 ($P < 0.05$). In addition, SVR was significantly associated with lower histological stage of liver disease ($P < 0.001$) and lower grade of steatosis in liver biopsy ($P = 0.001$). Patients ⩾

Table 2 Distribution of 56 genotype-1 and 40 genotype-3-infected patients by demographic, host and viral factors with respect to response to hepatitis C virus therapy (mean ± SD) *n* (%)

Variable	Genotype 1 (*n* = 56)			Genotype 3 (*n* = 40)		
	Responders (*n* = 32)	Non-responders (*n* = 24)	*P* value	Responders (*n* = 30)	Non-responders (*n* = 10)	*P*-value
Gender			0.70			0.47
Male	15 (46.9)	10 (41.7)		13 (43.3)	6 (60.0)	
Female	17 (53.1)	14 (58.3)		17 (56.7)	4 (40.0)	
Age (yr)			0.17			0.15
≤ 40	23 (71.9)	13 (54.2)		29 (96.7)	8 (80.0)	
> 40	9 (28.1)	11 (45.8)		1 (3.3)	2 (20.0)	
Body mass index (kg/m^2)			0.28			0.42
< 25	18 (56.3)	10 (41.7)		23 (76.7)	6 (60.0)	
25-28	14 (43.7)	14 (58.3)		7 (23.3)	4 (40.0)	
Grade of hepatic steatosis			0.0001			0.72
Mild	22 (68.7)	5 (20.8)		12 (40.0)	3 (30.0)	
Moderate	10 (31.3)	12 (50.0)		13 (43.3)	4 (40.0)	
Severe	0 (0.0)	7 (29.2)		5 (16.7)	3 (30.0)	
Fibrosis score			0.001			0.04
1	17 (53.1)	3 (12.5)		16 (53.3)	1 (10.0)	
2	11 (34.4)	8 (33.3)		11 (36.7)	7 (70.0)	
3-4	4 (12.5)	13 (54.2)		3 (10.0)	2 (20.0)	
5-6	0	0		0	0	
HOMA-IR			0.01			0.01
< 2	11 (34.4)	4 (16.7)		19 (63.3)	1 (10.0)	
2-3	10 (31.2)	2 (8.3)		10 (33.3)	7 (70.0)	
> 3	11 (34.4)	18 (75.0)		1 (3.3)	2 (20.0)	
Leptin_baseline (ng/mL)	37.66 ± 10.39	49.67 ± 13.44	0.001	24.33 ± 7.98	28.20 ± 9.72	0.22
Ghrelin_baseline (ng/mL)	0.286 ± 0.167	0.190 ± 0.119	0.02	0.778 ± 0.654	0.564 ± 0.324	0.18
Leptin end of follow-up (ng/mL)	26.69 ± 11.77	41.50 ± 16.24	< 0.0001	23.27 ± 9.54	26.70 ± 6.82	0.30
Ghrelin end of follow-up (ng/mL)	0.456 ± 0.254	0.239 ± 0.213	0.001	0.420 ± 0.321	0.450 ± 0.261	0.79

HOMA-IR: Homeostasis model assessment insulin resistance.

Table 3 Leptin and ghrelin concentrations at baseline and end of follow-up in patients infected with hepatitis C virus genotypes-1 and -3 by response to therapy, as well as mixed effect model derived estimates of differences in mean scores (mean ± SD)

Variable	Responders	Non-responders	*P*-value for the adjusted difference[1]
Leptin			
Baseline (ng/mL)	30.99 (9.185)	38.43 (11.58)	NS
End of Follow-up (ng/mL)	23.76 (8.055)	34.10 (11.53)	0.01
Ghrelin			
Baseline (ng/mL)	0.532 (0.410)	0.377 (0.223)	0.01
End of Follow-up (ng/mL)	0.438 (0.288)	0.345 (0.237)	0.05

[1]Adjusted for both leptin and ghrelin concentrations. NS: Non-significant.

40 years old tended to be less responsive to therapy (P = 0.05) (Table 1). Further analysis by genotype showed that, in genotype-1 infected patients, SVR was associated with a lower grade of liver steatosis (P = 0.0001), mild fibrosis (P = 0.001), and absence of insulin resistance (P = 0.01) (Table 2). In genotype-3 infected patients, SVR was associated with stage of fibrosis (P = 0.04) and lower HOMA-IR at baseline (P = 0.01), but not to degree of steatosis (Table 2).

Leptin, ghrelin and response to treatment

Baseline leptin concentrations did not differ between patients who did and did not attain SVR, but were significantly lower after successful treatment in patients who attained SVR (P = 0.01) (Table 3). Among patients infected with HCV genotype-1, non-responders had significantly higher serum leptin concentrations, both at baseline (P = 0.001) and at the end of follow-up (P < 0.0001) than those who attained SVR (Table 2). Using mixed effect model analysis, we observed a statistically significant difference between baseline and follow-up leptin concentrations among genotype-1 infected patients who achieved SVR (P = 0.001), as well as a borderline significant difference among non-responders (P = 0.06) (Table 4).

Among patients infected with HCV genotype-3, however, there were no significant differences in leptin concentrations at baseline and at end of follow-up between those who did and did not achieve SVR (Table 2). Using mixed effect model analysis, leptin remained unchanged, both in responders (P = 0.51) and non-responders (P = 0.61) (Table 4).

Overall, patients who achieved SVR had higher serum ghrelin concentrations, both at baseline (P = 0.01) and at the end of follow up (P = 0.05), than patients who did not achieve SVR (Table 3). Genotype-1 infected patients who achieved SVR had statistically significant higher ghrelin concentrations at baseline (P = 0.02) and at the end

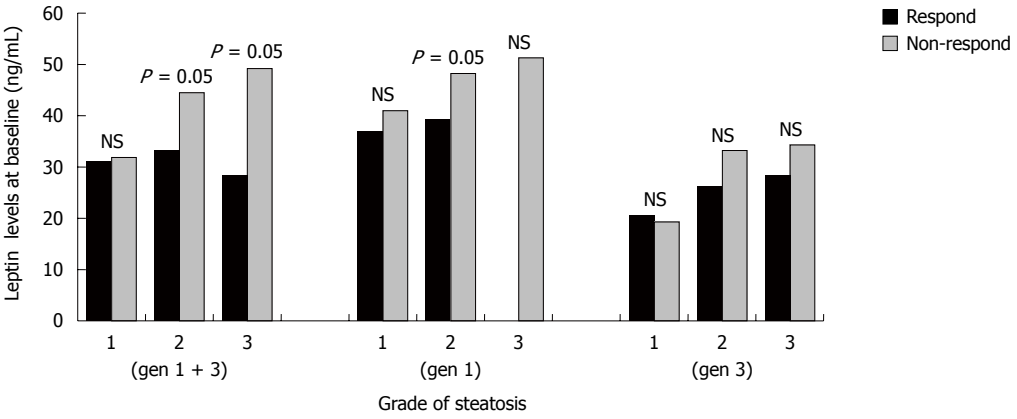

Figure 1 Correlation between leptin concentrations and steatosis in patients infected with hepatitis C virus genotypes-1 and -3 who did and did not achieve sustained virological response following treatment with PEGylated interferon a-2b or a-2a plus ribavirin. NS: Not significant.

Table 4 Leptin and ghrelin concentrations at baseline and at end of follow-up by response to treatment in patients with hepatitis C virus genotypes-1 and -3, as well as mixed effect model derived estimates of the differences in mean scores (mean ± SD)

Variable	Baseline	End of follow-up	*P*-value for the adjusted difference[1]
Genotype 1 SVRs			
Leptin (ng/mL)	37.66 (10.39)	26.69 (11.77)	0.001
Ghrelin (ng/mL)	0.286 (0.167)	0.456 (0.254)	0.001
Genotype 1 non-SVRs			
Leptin (ng/mL)	49.67 (13.44)	43.58 (16.17)	0.060
Ghrelin (ng/mL)	0.190 (0.119)	0.239 (0.213)	0.320
Genotype 3 SVRs			
Leptin (ng/mL)	24.33 (7.98)	23.27 (9.54)	0.510
Ghrelin (ng/mL)	0.778 (0.654)	0.420 (0.321)	0.001
Genotype 3 non-SVRs			
Leptin (ng/mL)	28.20 (9.72)	26.70 (6.82)	0.610
Ghrelin (ng/mL)	0.564 (0.324)	0.450 (0.261)	0.470

[1]Adjusted for both leptin and ghrelin concentrations.

of treatment ($P = 0.001$) than nonresponders (Table 2), with responders showing significantly higher ghrelin concentrations at end of treatment than at baseline ($P = 0.001$) (Table 4). In contrast, ghrelin concentrations in genotype-3 infected patients did not differ between responders and nonresponders, both at baseline and at the end of treatment (Table 2). Mixed effect model analysis showed that ghrelin concentrations were significantly lower at the end of treatment than at baseline in patients who achieved SVR ($P = 0.001$), but not in non-responders ($P = 0.47$) (Table 4).

Leptin, ghrelin and steatosis

Overall, steatosis grade at baseline was higher in non-responders than in patients who achieved SVR, with steatosis grade at baseline being significantly greater as leptin concentrations increased, a difference more obvious in patients with moderate and severe steatosis ($P = 0.05$). This correlation was also observed in genotype-1 non-responders, but not in genotype-3 non-responders or in patients of either genotype who achieved SVR (Figure 1).

Ghrelin concentration at baseline was higher in responders of both genotypes with moderate ($P = 0.05$) and severe ($P = 0.001$) steatosis. In non-responders, however, there was no significant correlation between the grade of steatosis and ghrelin concentrations. In genotype-1 infected patients, both in responders and non-responders, ghrelin concentrations decreased significantly as the grade of steatosis increased ($P = 0.01$), and responders with genotype-3 and moderate or severe steatosis had significantly higher serum concentrations of ghrelin ($P = 0.01$) (Figure 2). A strong correlation between the severity of steatosis and higher viral load at baseline was observed in patients infected with HCV genotype-3 ($P = 0.01$), but not in those infected with HCV genotype-1 (P = NS) (Figure 3).

Multivariate analysis

Using multivariate logistic regression analysis, we found that higher leptin concentrations at baseline were significantly associated with non-response to therapy in patients infected with HCV genotype-1, but not HCV genotype-3. There were no significant associations between response to treatment and ghrelin concentrations in patients of either genotype. Furthermore, HCV genotype-1 infected with moderate or severe steatosis, as well as those with more severe fibrosis, were less likely to respond to therapy (Table 5). In patients with genotype-3, however, there were no significant associations between response to treatment and steatosis, fibrosis, or leptin or ghrelin concentrations, both at baseline and at end of treatment. Only patients with higher levels of HOMA (IR) seemed less likely to respond to therapy (Table 6).

DISCUSSION

The mechanism by which HCV induces steatosis remains unclear. Steatosis in patients infected with the non-3 genotype has been associated with increased BMI, visceral obesity, increased cholesterol and triglyceride concentra-

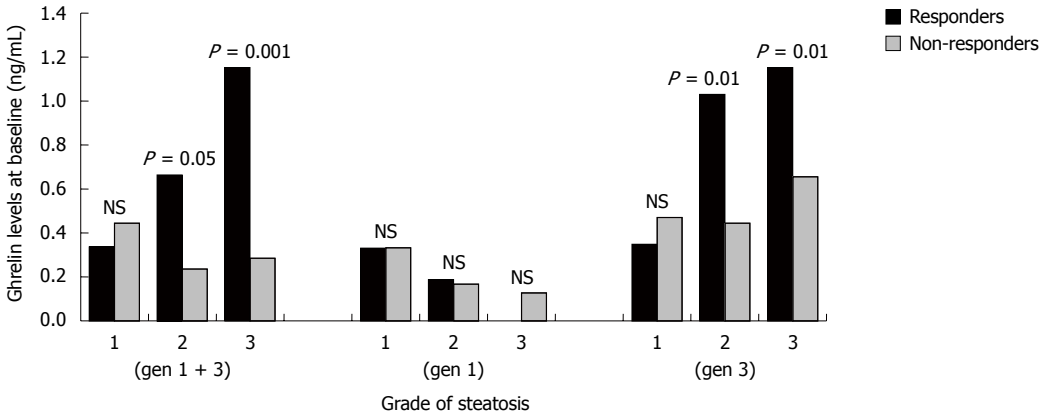

Figure 2 Correlation between ghrelin concentrations levels and steatosis in patients infected with hepatitis C virus genotypes11 and -3 who did and did not achieve sustained virological response following treatment with PEGylated interferon a-2b or a-2a plus ribavirin. NS: Not significant.

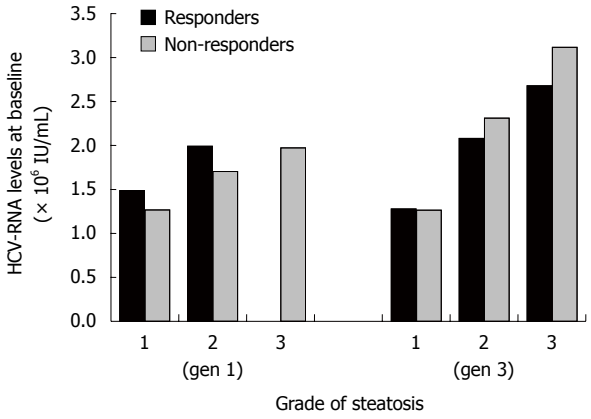

Figure 3 Correlation between viral load and steatosis in patients infected with hepatitis C virus genotypes-1 and -3 who did and did not achieve sustained virological response following treatment with PEGylated interferon a-2b or a-2a plus ribavirin. HCV: Hepatitis C virus.

tions, insulin resistance, metabolic syndrome, diabetes, alcohol consumption, and increased sensitivity of the liver to oxidative stress or cytokine-mediated injury[17,18].

Leptin is a putative link between HCV infection and steatosis[19]. Although a high incidence of hyperleptinemia has been observed in HCV infected patients with liver steatosis[10,20], the underlying mechanism promoting this effect remains undefined. Leptin may increase insulin resistance and fatty acid concentrations in the liver, leading to enhanced lipid peroxidation and promoting steatosis[5]. Leptin may also induce the release of cytokines, such as TNF-α, INF-γ, IL-18, and TGF-β1, which are involved in the pathogenesis of both liver steatosis and fibrosis[8]. In steatosis, activated hepatic stellate cells, but not quiescent cells, can express leptin[21].

Our results clearly show that, in HCV infected patients with liver steatosis, serum leptin levels tend to increase as the grade of steatosis worsens. This finding is significant, especially in genotype-1 patients, suggesting that leptin increases during infection as a part of the host immune response, and may contribute to the development of steatosis. Although steatosis is more common

Table 5 Multiple logistic regression derived odds ratios and 95% confidence intervals for response to hepatitis C virus therapy among patients infected with hepatitis C virus genotype-1 with respect to hepatic steatosis, and leptin and ghrelin concentrations at baseline and at end of follow-up

Variable	Category or increment	ORs	95% CIs	*P*-value
At baseline				
Hepatic steatosis	1 level more	0.12	0.02-0.66	0.01
Leptin	10 ng/mL more	0.43	0.22-0.83	0.01
Ghrelin	0.1 ng/mL more	1.11	0.67-2.01	0.63
HOMA-IR	1 level more	1.34	0.42-4.31	0.63
Alternatively introduced variables				
Fibrosis	1 level more	0.36	0.13 -0.96	0.04
Leptin	10 ng/mL more	0.45	0.24-0.86	0.02
Ghrelin	0.1 ng/mL more	1.35	0.82-2.45	0.26
HOMA-IR	1 level more	0.96	0.33-2.79	0.94
At end of follow-up				
Hepatic steatosis	1 level more	0.13	0.02-0.89	0.04
Leptin	10 ng/mL more	0.38	0.18-0.81	0.01
Ghrelin	0.1 ng/mL more	1.22	0.74-2.01	0.41
HOMA-IR	1 level more	1.98	0.54-7.30	0.30
Alternatively introduced variables				
Fibrosis	1 level more	0.30	0.10-0.93	0.04
Leptin	10 ng/mL more	0.34	0.16-0.72	0.01
Ghrelin	0.1 ng/mL more	1.22	0.74-1.82	0.47
HOMA-IR	1 level more	1.28	0.43-3.79	0.66

CI: Confidence interval; HOMA-IR: Homeostasis model assessment insulin resistance; ORs: Odds ratios.

and more severe in patients infected with HCV genotype-3[17,18], we found that leptin concentrations were not correlated with either the grade of steatosis or response to treatment. Structural and nonstructural proteins of HCV genotype-3 may directly cause steatosis by provoking oxidative stress[6,22,23]. Alternatively, the viral core protein may target microsomal triglyceride transfer protein activity, modifying very low density lipoprotein assembly in, and secretion by hepatocytes[24,25]. The core protein may also affect the cytoplasmic domain of members of the TNF receptor family or act directly on the mitochondria, leading to increased oxidative stress and lipid peroxidation[26]. In patients infected with HCV genotype-3,

Table 6 Multiple logistic regression derived odds ratios and 95% CIs for response to hepatitis C virus therapy among patients infected with hepatitis C virus genotype-3 with respect to hepatic steatosis, and leptin and ghrelin concentrations at baseline and at end of follow-up

Variable	Category or increment	ORs	95% CIs	*P*-value
At baseline				
Hepatic steatosis	1 level more	1.04	0.19-5.55	0.97
Leptin	10 ng/mL more	0.64	0.18-2.24	0.49
Ghrelin	0.1 ng/mL more	1.11	0.90-1.49	0.32
HOMA-IR	1 level more	0.13	0.02-0.68	0.02
Alternatively introduced variables				
Fibrosis	1 level more	0.16	0.02-0.11	0.06
Leptin	10 ng/mL more	1.22	0.37-4.08	0.74
Ghrelin	0.1 ng/mL more	1.22	0.90-1.65	0.16
HOMA-IR	1 level more	0.16	0.03-0.83	0.03
At end of follow-up				
Hepatic steatosis	1 level more	1.77	0.39-7.95	0.46
Leptin	10 ng/mL more	0.40	0.10-1.55	0.18
Ghrelin	0.1 ng/mL more	1.00	0.74-1.22	0.76
HOMA-IR	1 level more	0.10	0.02-0.59	0.01
Alternatively introduced variables				
Fibrosis	1 level more	0.40	0.08-1.52	0.16
Leptin	10 ng/mL more	0.70	0.22-2.18	0.54
Ghrelin	0.1 ng/mL more	1.00	0.74-1.35	0.85
HOMA-IR	1 level more	0.14	0.03-0.71	0.02

CI: Confidence interval; HOMA-IR: Homeostasis model assessment insulin resistance; ORs: Odds ratios.

we found that the grade of steatosis was correlated with higher viral load at baseline, in agreement with the direct "steatogenic" effect of this genotype[6].

Although ghrelin is important in food intake, energy balance, and the regulation of the growth hormone releasing mechanism[15], its role in hepatic disease has not been extensively evaluated to date. Increased serum ghrelin concentrations have been reported in patients with cirrhosis and hepatocellular carcinoma, suggesting that this adipokine may be involved in the anorexia-cachexia syndrome during the terminal stages of liver diseases[27]. Data on ghrelin concentrations in patients with CHC are limited[28].

We found that genotype-1 responders had higher serum ghrelin concentrations at baseline than non-responders, and that its concentration increased significantly in the former at the end of treatment, indicating that ghrelin may prevent or reduce steatosis by negatively regulating leptin. This may enhance the likelihood of SVR, since responders also have lower baseline leptin concentrations. In genotype-3 infected patients, however, ghrelin may be considered an independently acting factor, based on our finding that responders with moderate and severe steatosis had high ghrelin concentrations at baseline and that these concentrations were reduced significantly after treatment. In contrast, no significant differences were observed in non-responders and there were no correlations with leptin concentrations.

Our findings are in accordance with previous reports, which found that steatosis was an independent negative predictor of response to antiviral therapy[29-32]. We also found that genotype 1 patients with elevated leptin concentrations before treatment had a lower likelihood of achieving SVR, irrespective of their viral load. This observation is in keeping with the role of leptin as a suppressor of cytokine signaling 3 in the liver[33], or as a factor that inhibits INF signaling.

In conclusion, our results suggest that the extent of hepatic steatosis, in addition to the stage of fibrosis and the viral genotype, may affect the likelihood of SVR in CHC patients. Leptin appears to contribute to the pathogenesis of steatosis, and we found that elevated serum leptin concentration may be an independent predictor of SVR in HCV genotype-1 infected patients. Increase ghrelin concentrations after successful treatment in genotype 1 patients indicate that this peptide plays a role in the achievement of SVR. Further investigations are needed to determine whether ghrelin acts as a downstream mediator of leptin and to assess the influence of ghrelin on liver steatosis and HCV infection.

COMMENTS

Background

Steatosis is a frequent histopathological feature in patients with chronic hepatitis C (CHC). Leptin and ghrelin are involved in body fat regulation and metabolism. Higher serum leptin concentrations have been associated with steatosis, but less is known about leptin and ghrelin concentrations in patients with CHC and steatosis or the effect of these peptides on response to treatment.

Research frontiers

Leptin is a putative link between hepatitis C virus (HCV) infection and steatosis in HCV genotype-1 infected patients; however, the underlying mechanism remains undefined. Increased ghrelin concentrations have been reported in patients with cirrhosis and hepatocellular carcinoma, but its role in hepatic steatosis has not been extensively evaluated. Steatosis is an independent negative predictor of response to antiviral therapy. Our results clearly show that, in HCV infected patients with steatosis, serum leptin levels tend to increase as the grade of steatosis worsens. Non-responding genotype-1 infected patients have elevated leptin at baseline and genotype-1 and -3 responders have higher ghrelin concentrations at baseline. In genotype-3 infected patients, neither the degree of steatosis nor leptin concentration had any effect on response to treatment.

Innovations and breakthroughs

Several studies have highlighted the importance of steatosis and hyperleptinemia in the achievement of sustained virological response (SVR), but this study is the first to find genotype-dependent associations between the degree of steatosis and leptin and ghrelin concentrations. These findings show the significance of baseline leptin and ghrelin concentrations in the achievement of SVR, as well as the impact of antiviral treatment on serum leptin and ghrelin levels.

Applications

These findings suggest that serum leptin concentrations may be an independent negative predictor of SVR in HCV genotype-1 infected patients with steatosis; the role of ghrelin requires be further investigation.

Terminology

Leptin, the ob gene product, is expressed mainly by adipose tissue, although it is expressed in other organs, including the liver. Leptin is important for body fat regulation and metabolism. Ghrelin, a peptide that acts as an endogenous ligand for the growth hormone secretatog receptor, is involved in energy metabolism, food intake and glucose homeostasis.

Peer review

The research study has an important outcome and could be further strengthened by exploring the existing data for an effect of sex on these parameters, if any.

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BRIEF ARTICLE

In vitro effect of pantoprazole on lower esophageal sphincter tone in rats

Mustafa Duman, Mahmut Özer, Enver Reyhan, Yeliz Demirci, Ali E Atıcı, Tahsin Dalgıç, Erdal B Bostancı, Ece Genç

Mustafa Duman, Enver Reyhan, Ali E Atıcı, Tahsin Dalgıç, Erdal B Bostancı, Department of Gastrointestinal Surgery, Kartal Kosuyolu Education and Research Hospital, Cevizli, Kartal, 34846 Istanbul, Turkey
Mahmut Özer, Yeliz Demirci, Ece Genç, Department of Pharmacology, Medical School, Yeditepe University, Atasehir, 34755 Istanbul, Turkey
Author contributions: Duman M, Özer M and Demirci Y performed the majority of the experiments; Reyhan E, Atıcı AE and Dalgıç T conducted the statistical analysis and interpreted the results; Bostancı EB provided the necessary chemicals and contributed to the design of the experiment; Genç E contributed to the design of the study and writing of the manuscript.
Correspondence to: Ece Genç, Professor, Department of Pharmacology, Medical School, Yeditepe University, Kayışdağı, Ataşehir, 34755 Istanbul, Turkey. egenc@yeditepe.edu.tr
Telephone: +90-216-5780528 Fax: +90-216-5780575
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Abstract

AIM: To investigate the *in vitro* effects of pantoprazole on rat lower esophageal sphincter (LES) tone.

METHODS: Rats weighing 250-300 g, provided by the Yeditepe University Experimental Research Center (YÜDETAM), were used throughout the study. They were anesthetized before decapitation. LES tissues whose mucosal lining were removed were placed in a standard 30-mL organ bath with a modified Krebs solution and continuously aerated with 95% oxygen-5% carbon dioxide gas mixture and kept at room temperature. The tissues were allowed to stabilize for 60 min. Subsequently, the contractile response to 10^{-6} mol/L carbachol was obtained. Different concentrations of freshly prepared pantoprazole were added directly to the tissue bath to generate cumulative concentrations of 5×10^{-6} mol/L, 5×10^{-5} mol/L, and 1.5×10^{-4} mol/L. Activities were recorded on an online computer *via* a 4-channel transducer data acquisition system using the software BSL PRO v 3.7, which also analyzed the data.

RESULTS: Pantoprazole at 5×10^{-6} mol/L caused a small, but statistically insignificant, relaxation in the carbachol-contracted LES (2.23% *vs* 3.95%). The 5×10^{-5} mol/L concentration, however, caused a significant relaxation of 10.47% compared with the control. 1.5×10^{-4} mol/L concentration of pantoprazol caused a 19.89% relaxation in the carbachol contracted LES ($P < 0.001$).

CONCLUSION: This is the first study to demonstrate that pantoprazole has a relaxing effect in isolated LESs. These results might have significant clinical implications for the subset of patients using proton pump inhibitors who do not receive full symptomatic alleviation from gastroesophageal reflux disease.

Key words: Pantoprazole; Lower esophageal sphincter, Gastroesophageal reflux disease

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INTRODUCTION

The esophagogastric junction is located between the esophagus and the stomach. The high-pressure zone at the junction between the esophagus and the stomach is composed of the lower esophageal sphincter (LES) and

the crural diaphragm[1,2]. Circular smooth muscle from the esophageal body generates little if any tone at rest, whereas the circular smooth muscle of LES is characterized by a spontaneously generated basal tone that prevents the reflux of gastric contents into the esophagus[3,4]. The basal tone of the LES is primarily myogenic in origin, but can be modulated by both neural and hormonal factors[5]. In response to esophageal distension and swallowing, the LES relaxes[6]. The abnormal dynamics of LES function are considered to be the most important factors in the pathogenesis of gastroesophageal reflux disease (GERD)[7-10]. GERD is described as the reflux of gastric contents into the esophagus leading to reflux symptoms and esophagitis sufficient to affect patient wellbeing and/or induce complications. These complications range from esophagitis to adenocarcinoma of the distal esophagus. Furthermore it may cause extra esophageal symptoms, such as cough, laryngitis and asthma[11,12]. GERD is a highly prevalent in the general population, affecting up to 10%-30% of the adult population in western countries[13].

Pharmacological therapy is necessary in the majority of patients. GERD is currently treated with acid suppressing drugs, such as proton pump inhibitors (PPIs); however, for those refractory to pharmacological treatment, surgery is often recommended[13,14]. PPIs are the mainstay of medical management for GERD[11]. They have been widely used since the 1980s and have been considered as ideal drugs because of their highly specific pharmacologic actions[15,16]. Although PPIs have been used as a common treatment modality in GERD, there is a lack of experimental studies of their effects on isolated LES preparations.

The aim of this study was to investigate the effect of a PPI, pantoprazole, on the tone of the isolated rat LES preparations contracted by carbachol. This study provides a significant contribution to this somewhat ignored area of research.

MATERIALS AND METHODS

The experimental protocol was approved by the Ethical Committee of Yeditepe University Experimental Medicine Research Institute and the use of animals was in compliance with US National Institutes of Health Guide for Care and Use of Laboratory Animals.

Sixteen rats weighing 250-300 g, provided by the Yeditepe University Experimental Research Center (YÜDETAM), were used throughout the study. They were kept in plexiglass cages in a room whose temperature and humidity were controlled with 12-h light/dark cycle, and had free excess to food and water.

Rats were anesthetized with a combination of 10 mg/kg xylazine HCl (Rompun® 2%, Bayer HealthCare AG, Leverkusen-Germany) and 100 mg/kg ketamine HCl (Ketasol® 10%, Richter Pharma AG, Weis-Austria) before decapitation.

A midline incision was performed to open up the abdominal cavity and the LES was carefully dissected out and placed in a petri dish containing Krebs solution at room temperature. Thereafter, the mucosal lining was removed and the sphincteric muscle was set up, as a ring segment 2 mm in width, in Krebs solution contained in a standard 30-mL organ bath. The modified Krebs solution comprised NaCl, 118.07 mmol/L; KCl, 4.69 mmol/L; $CaCl_2$, 2.52 mmol/L; $MgSO_4$, 1.16 mmol/L; KH_2PO4, 1.2 mmol/L; $NaHCO_3$, 25 mmol/L, and glucose, 11.10 mmol/L. Krebs solution was continuously aerated with 95% oxygen-5% carbon dioxide gas mixture and kept at 37 ± 0.5 ℃ throughout the experimental period. The tissues were tied to stainless steel hooks at one end of the organ bath; the other end was connected to a force transducer (FDT 05, May, COMMAT Iletisim Co, Ankara-Turkey) under a resting tension of around 1 g. LES ring activities were recorded on an online computer *via* a 4-channel transducer data acquisition system (MP35, BIOPAC Systems Inc. Goleta, CA, United States) using the software BSL PRO v 3.7 (BIOPAC Systems Inc. Goleta, CA, United States), which also analyzed the data.

The following compounds were used: carbachol chloride (Carbamylcholine chloride, Sigma-Aldrich Chemical Co. St. Louis, MO, United States) and pantoprazole (Pantoprazole sodium, Dr. Reddy's Laboratories Ltd. Hyderabad-India). Solutions were prepared daily in distilled water and kept at 4 ℃ during the experiments. Pantoprazole was treated with 1 mol/L HCl and its pH was adjusted to 4.0 before application to the organ bath. Following a 60-min equilibration period for stabilization, the contractile response to carbachol was obtained by application of a single dose of charbachol to a final concentration of 10^{-6} mol/L in the organ bath. After the contractions reached a plateau, concentration-response relationships for pantoprazole (final organ bath concentrations of 5×10^{-6} mol/L, 5×10^{-5} mol/L and 1.5×10^{-4} mol/L, with 15 min allotted between each dose) were obtained in a cumulative manner. (These doses were calculated to be the equivalent of Human doses for the rats). Control experiments were also run with only acidified distilled water added to the organ bath. The relaxations were quantified by integrating the area under the curve for each concentration and control group. At the end of the each experiment, tissues were weighed and the final pH of the Krebs solution was measured.

Statistical analysis

For statistical evaluation, analysis of variance (One way ANOVA) was performed with the program SPSS for windows version 18 (SPSS Inc. Chicago, Illinois). Values of $P < 0.05$ were considered as statistically significant.

RESULTS

The experiment design is outlined in Figure 1. Pantoprazole caused dose dependent relaxation of the carbachol-contracted LES preparations. No such effect was observed in the control group (Figure 1B). The relaxations were quantified by integrating the area under the curve

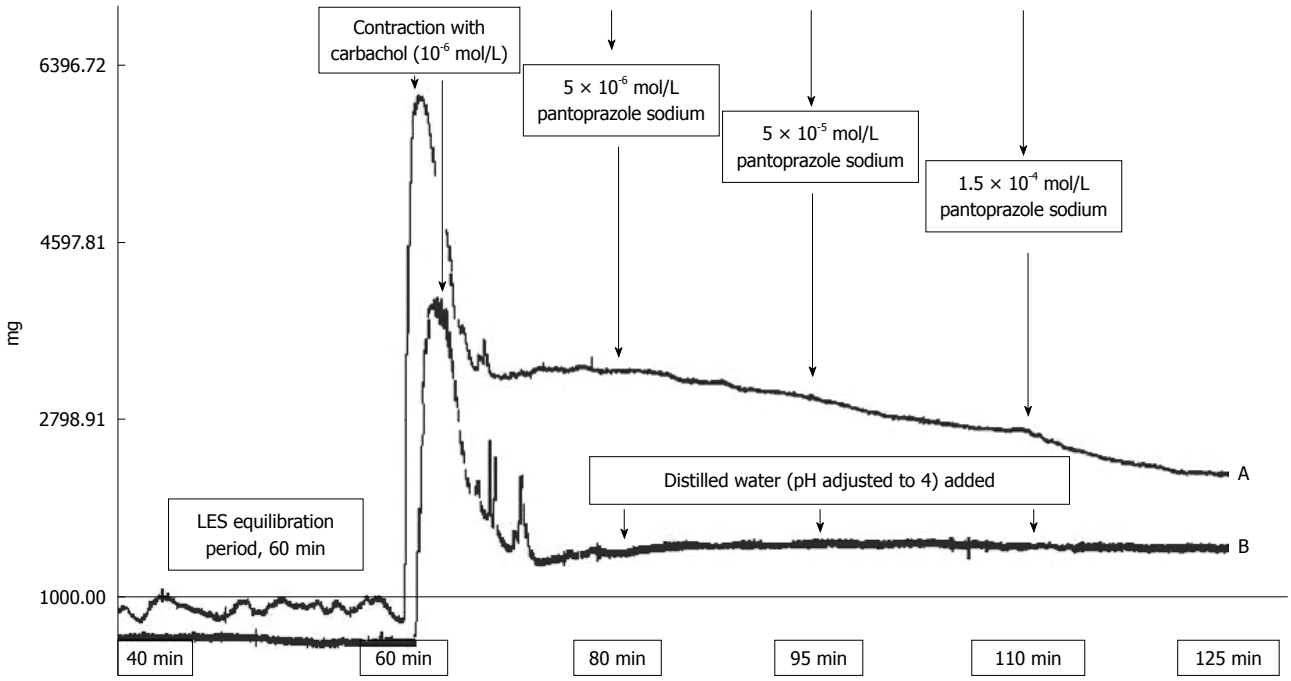

Figure 1 Outline of the experimental procedure. A: The tissues were allowed to stabilize for 60 min in Krebs-containing organ baths. Following that period, their contractile response to 10^{-6} mol/L carbachol was obtained. Pantoprazole was treated with 0.1 mol/L HCl and the pH of the drug solution was adjusted to 4.0. Different concentrations of pantoprazole were added directly to the tissue bath to generate cumulative concentrations of 5×10^{-6} mol/L, 5×10^{-5} mol/L and 1.5×10^{-4} mol/L. The relaxations were quantified by integrating area under the curve for each concentration; B: For the control experiments, acidified distilled water was added at the same time points. LES: Lower esophageal sphincter.

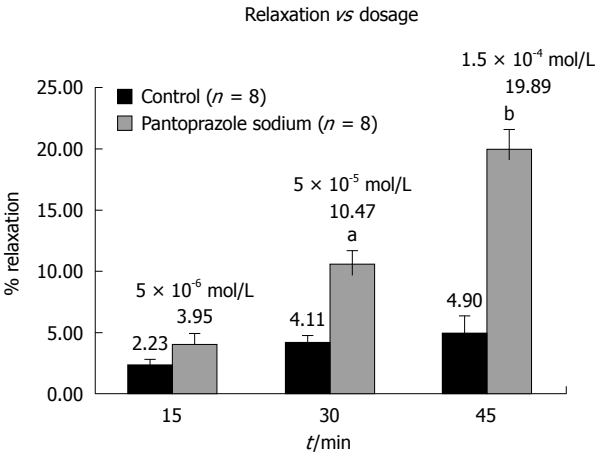

Figure 2 Relaxation *vs* Dosage. 5×10^{-5} mol/L and 1.5×10^{-4} mol/L pantoprazole sodium induced significant relaxation in lower esophageal sphincter preparations *in vitro* ($^{a}P < 0.05$ and $^{b}P < 0.001$). Each bar represents percent relaxation ± SEM for both control and experiment groups. Numbers in parentheses indicate the number of preparations used from different animals.

for each concentration.

The mean of integral values and percent relaxations of eight preparations were compared for statistical evaluation. As shown in Figure 2, application of pantoprazole sodium in a cumulative manner resulted in significant relaxations of LES preparations at 5×10^{-5} mol/L and 1.5×10^{-4} mol/L concentrations.

In the carbachol-contracted LES preparations 5×10^{-6} mol/L pantoprazole caused a 4% relaxation, while higher doses caused significant relaxations. Mean integral relaxation values were 4.11% ± 0.58% (SE) and 10.47% ± 1.2% (SE) for control and 5×10^{-5} mol/L pantoprazole, respectively ($P < 0.05$). Moreover, these values were 4.90% ± 1.4% (SE) and 19.89% ± 1.7% (SE) for control and 1.5×10^{-4} mol/L concentrations, respectively ($P < 0.001$) (Figure 2).

DISCUSSION

The aim of the present work was to assess the *in vitro* effects of pantoprazole on LES tone in rats. The reason why pantoprazole was chosen was the drug's frequent use in our Clinic. The major finding of our study was that pantoprazole caused a dose-dependent decrease in LES tone. This is the first study to demonstrate that pantoprazole has such an effect on isolated LES.

LES is an important specialized smooth muscle in the gastrointestinal tract and has been the subject of investigation by many authors[17-20]. GERD is a highly prevalent condition and is a major burden to society as well as the afflicted individual. Although numerous clinical studies have been conducted to clarify the mechanism of GERD, a clear consensus has not been reached. Regarding the pathophysiologies of GERD, decrease of LES basal tone and transient relaxations of the LES (TLOSRs) as a response to gastric distension[21], and excessive exposure of the esophagus to gastric acid, have been reported to be important[21-24].

GERD is, in most cases, successfully treated with PPIs, which have largely replaced Histamine H_2 receptor blockers because of their well documented efficacy

and because they are well tolerated, with relatively few serious adverse effects. However, a significant number of patients do not receive full symptomatic relief[25,26]. Thus, a significant question that has to be addressed is why some GERD patients are resistant to the effects of PPIs? In addition to neonates and infants who respond poorly to PPIs[27], some adults do not benefit from them either. In a study conducted by Hemmink *et al*[28] in 2008, there were fewer acid reflux episodes in patients on PPI therapy; however, weak acidic reflux episodes increased under the influence of PPIs. The total number of reflux episodes, on the other hand, was not affected. In addition to these, there have been recent papers regarding the adverse effects of PPIs[29,30]. Corley *et al*[31] showed that PPIs are associated with hip fractures among at-risk patients. They can also cause neutropenia in some patients[32]. Acid suppression also causes nosocomial *Clostridium* difficile infections in a dose-dependent manner[33].

These results point out the necessity of developing novel approaches for GERD. Coman *et al*[34] demonstrated the significance of adding prokinetic drugs to the treatment of GERD, in a study conducted on 1118 patients. The effects of specific GABA B receptor agonists have also been studied[35]. Drugs that reduce TLOSRs have also been suggested as pharmacological agents for GERD[36].

At present, the mechanism of the pantoprazole-induced relaxation of LESs can only be speculated. However, there are 2 types of muscles in the LES, circular muscle and sling muscle. Circular smooth muscle is tonically contracted with cholinergic stimulation. In response to swallowing, a peristaltic contraction travels down the length of the esophagus and the LES relaxes.

Nitric oxide (NO)[37,38] and vasoactive intestinal polypeptide (VIP)[39,40] are proposed as neurotransmitters that control relaxation. Both VIP and NO can be released from esophageal nerves with an appropriate stimulus, and NO synthase and VIP are found in myenteric neurons that innervate the circular smooth muscle of the esophagus. Sarioglu *et al*[41], showed the relaxant effect of omeprazole in rabbit corpus cavernosum *in vitro*. They concluded that the relaxant effect is probably due to the L-type Ca^{2+} channel blockage by omeprazole. We can speculate that a similar mechanism is responsible for the effect of pantoprazole on LESs.

The present study is the first to demonstrate a dose-dependent decrease in the carbachol-induced contraction of the LES by pantoprazole. Although this finding has been observed in an isolated tissue, it might have some clinical correlates and might help to understand why the treatment of GERD requires additional pharmacological interventions.

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COMMENTS

Background

Gastroesophageal reflux disease (GERD) is a highly prevalent condition in the general population, affecting up to 10%-30% of the adult population in Western countries[13]. The incidence of GERD is rising very rapidly due to the stressful lives. New approaches are necessary for its treatment.

Research frontiers

Not all patients benefit from the proton pump inhibitors (PPIs) that are frequently used for the treatment of GERD. The authors conducted an experiment to investigate the effects of these drugs on isolated rat lower esophageal sphincters (LESs). There was a dose dependent decrease in LES tone.

Innovations and breakthroughs

The study conducted is the first to demonstrate the effects of pantoprazole on the isolated LESs of rat, including the dose dependent decrease in the tone of LESs under the effect of the drug.

Applications

The study suggests that doctors should be cautious about long-term use of PPIs for the treatment of GERD.

Peer review

This paper should be of interest to a broad readership including gastroenterologists, pharmacologists, and physicians of internal medicine. It is also of interest to gastrointestinal surgeons. This paper is very interesting and is an important study to publish.

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BRIEF ARTICLE

Autofluorescence imaging endoscopy for identification and assessment of inflammatory ulcerative colitis

Taro Osada, Atsushi Arakawa, Naoto Sakamoto, Hiroya Ueyama, Tomoyoshi Shibuya, Tatsuo Ogihara, Takashi Yao, Sumio Watanabe

Taro Osada, Naoto Sakamoto, Tomoyoshi Shibuya, Tatsuo Ogihara, Sumio Watanabe, Department of Gastroenterology, Juntendo University School of Medicine, 113-8421 Tokyo, Japan
Atsushi Arakawa, Hiroya Ueyama, Takashi Yao, Department of Human Pathology, Juntendo University School of Medicine, 113-8421 Tokyo, Japan
Author contributions: Osada T conceived and designed the study; Osada T, Arakawa A, Sakamoto N, Ueyama H, Shibuya T and Ogihara T collected the data; Osada T, Yao T and Watanabe S interpreted the data; Osada T wrote the manuscript; all authors discussed the results and commented on the manuscript; Watanabe S gave final approval of this article.
Correspondence to: Taro Osada, MD, PhD, Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, 113-8421 Tokyo, Japan. otaro@juntendo.ac.jp
Telephone: +81-3-58021060 Fax: +81-3-38138862
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Abstract

AIM: To validate the clinical relevance of autofluorescence imaging (AFI) endoscopy for the assessment of inflammatory ulcerative colitis (UC).

METHODS: A total of 572 endoscopic images were selected from 42 UC patients: 286 taken with white light imaging (WLI) and 286 with AFI from the same sites. WLI images were assessed for overall mucosal inflammation according to Mayo endoscopic subscore (MES), and for seven characteristic endoscopic features. Likewise, AFI photographs were scored according to relative abundance of red, green and blue color components within each image based on an RGB additive color model. WLI and AFI endoscopic scores from the same sites were compared. Histological evaluation of biopsies was according to the Riley Index.

RESULTS: Relative to red (r = 0.52, P < 0.01) or blue (r = 0.56, P < 0.01) color component, the green color component of AFI (r = -0.62, P < 0.01) corresponded more closely with mucosal inflammation sites. There were significant differences in green color components between MES-0 (0.396 ± 0.043) and MES-1 (0.340 ± 0.035) (P < 0.01), and between MES-1 and ⩾ MES-2 (0.318 ± 0.037) (P < 0.01). The WLI scores for "vascular patterns" (r = -0.65, P < 0.01), "edema" (r = -0.62, P < 0.01), histology scores for "polymorphonuclear cells in the lamina propria" (r = -0.51, P < 0.01) and "crypt architectural irregularities" (r = -0.51, P < 0.01) showed correlation with the green color component of AFI. There were significant differences in green color components between limited (0.399 ± 0.042) and extensive (0.375 ± 0.044) (P = 0.014) polymorphonuclear cell infiltration within MES-0. As the severity of the mucosal inflammation increased, the green color component of AFI decreased. The AFI green color component was well correlated with the characteristic endoscopic and histological inflammatory features of UC.

CONCLUSION: AFI has application in detecting inflammatory lesions, including microscopic activity in the colonic mucosa of UC patients, based on the green color component of images.

Key words: Autofluorescence imaging endoscopy; Endoscopic activity; Histological activity; Microscopic inflammation; Green color component

Peer reviewers: Arjuna De Silva, Professor in Medicine, Department of Medicine, Faculty of Medicine, University of Kelaniya, PO Box 6, Tallagolla Road, 11010 Ragama, Sri Lanka; Jukka-Pekka Mecklin, MD, PhD, Professor, Surgeon-in-Chief, Department of Surgery, Jyvaskyla Central Hospital, 40620 Jyvaskyla, Finland

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INTRODUCTION

Ulcerative colitis (UC) activity is routinely assessed by a combination of clinical, endoscopic, and histological findings. However, systematic measurement of disease activity in UC patients is essential for determining the efficacy of treatment interventions. Endoscopy in particular is the most immediate and objective method for evaluating colonic mucosal damage. Furthermore, the severity of mucosal inflammation in UC is evaluated based on several disease activity indices, but there is no validated gold standard. To standardize the assessment of disease activity for clinical trials, in 1987, Schroeder *et al*[1] described the Mayo Score, which includes endoscopic findings. This index includes the factors "erythema", "vascular pattern", "friability", "erosion" and "spontaneous bleeding". "Edema" and "granularity" are also factored in other endoscopic indices, such as Matts' endoscopic activity index. These features are considered to be characteristic endoscopic findings in UC. However, inter- and intra-observer variations, and variations depending on the observer's experience in the endoscopic assessment of UC based on conventional endoscopy, have been reported[2].

Histological activity generally shows strong correlation with the activity evaluated by endoscopy. However, microscopic evaluations reflect clinical symptoms more accurately than endoscopic evaluations[3]. Endoscopic appearance alone tends to underestimate the severity of disease activity as compared with histological evaluation, and is not able to detect microscopic activity[4-6]. The features of mucosal lesions in UC are considered to include the presence of polymorphonuclear leukocytes in the lamina propria, the formation of crypt abscesses, ulcers and mononuclear cell infiltrate in the lamina propria together with crypt architectural irregularities[7]. In the Riley Index, 6 histological features of UC are factored and each feature is graded on a four-point scale[8]; this index has been applied in some clinical trials.

Autofluorescence imaging (AFI) videoendoscopy produces real-time pseudocolor images based on tissue autofluorescence emitted by excitation of endogenous tissue fluorophores, which mainly consist of collagen type- I [9]. If the change of hue on AFI indicated the extent and severity of UC activity, the analysis would be an objective and reproducible method regardless of the observer's experience. With this in mind, the aim of this study was to evaluate the correlations between the results of analysis of the change in hue on AFI with the results of white light imaging (WLI), together with histological findings on UC activity, to better understand the clinical relevance of AFI.

MATERIALS AND METHODS

Selection of images

This study included 42 patients with a diagnosis of UC, 31 male and 11 female, aged 36.2 ± 11.0 (mean ± SD) years who underwent colonoscopic examinations with a CF-FH260AZL/I colonovideoscope (Olympus Inc., Tokyo Japan). This endoscopic system comprised a high resolution white-light endoscope with optical zoom (magnification 75 ×) equipped with an AFI and narrow band imaging (NBI). The same sites were observed by WLI and AFI. Stored images were retrieved from the computerized database of the Endoscopy Centre at Juntendo University Hospital (Tokyo, Japan) by using an endoscopic filing system, Scope Reader M1 (AZ Co., Ltd., Sendai, Japan). A total of 572 endoscopic images, 286 with WLI and 286 with AFI, from the same sites where tissue biopsy specimens were subsequently taken were selected. The diagnosis of UC was based on the following criteria: history of recurrent bloody and mucous stools, endoscopic findings of ulceration, mucosal friability, loss of vascular architecture or presence of diffuse lesions increasing in severity towards the rectum, and no evidence of pathogenic micro-organisms in stool cultures. The images could be downloaded from the server in JPEG (Joint Photographic Experts Group) format without loss of quality. The file size of each downloaded image was about 100 kilobytes, with a pixel array of 640 × 480, and in 24-bit color.

Examination of images

Initially, all endoscopic examinations were done by one expert endoscopist. Subsequently, the images of WLI were provided for examination by two expert endoscopists (NS and TO), who discussed their assessments and reached a consensus view on the endoscopic activity. Each of these expert endoscopists had performed more than 5000 colonoscopy procedures, and was familiar with UC disease activity *via* endoscopy. Each WLI image was examined and graded for overall mucosal inflammation according to the Mayo endoscopic subscore (MES), scored on a scale of 0 to 3 (Table 1), and for seven endoscopic characteristic features of UC including vascular patterns, erythema, edema, granularity, erosions, ulcers and friability, scored on a scale of 0 to 10 (Table 2).

The optical findings with AFI colonoscopy were displayed as pseudocolored images, and laid over a composite image on the video display. All computer and video displays used the RGB additive color model in which red, green and blue lights are added together in various ways to produce a broad array of colors (256 × 256 × 256 = 16 million). AFI images were evaluated by analyzing the hue, and scored for the relative abundance of red [R/(R + G + B)], green [G/(R + G + B)] and blue

Table 1 Endoscopic scoring system scored from 0 to 3 (Mayo endoscopic subscore)

Score	
0	Normal or inactive disease
1	Mild disease (erythema, decreased vascular pattern, mild friability)
2	Moderate disease (marked erythema, absent vascular pattern, friability, erosions)
3	Severe disease (spontaneous bleeding, ulceration)

Table 2 Seven endoscopic features scored from 0 to 10 points

Vascular pattern	0 (normal) to 10 (absent)
Erythema	0 (absent) to 10 (marked)
Edema	0 (absent) to 10 (marked)
Granularity	0 (absent) to 10 (marked)
Erosions	0 (absent) to 10 (multiple)
Ulcerations	0 (absent) to 10 (multiple)
Friability	0 (absent) to 10 (marked)

Table 3 Histological scoring system (Riley index)

1	Round cells in the lamina propria
2	Polymorphonuclear cells in the lamina propria
3	Crypt abscesses
4	Mucin depletion
5	Surface epithelial integrity
6	Crypt architectural irregularities

Each item is scored from 0 (absent) to 3 points (severe).

(B/[R+G+B]) color components based on the RGB color model which involves the use of Adobe® Photoshop® Elements® 5.0. The color of the region on AFI, where tissue biopsy specimens were subsequently taken, was divided into red, green and blue color components. Although the actual count of each color varied as its brightness varied, the abundance of the actual count was fairly constant (Figure 1). All AFI and WLI images were displayed anonymously to the observers and revealed neither clinical data nor the date on which the images were taken.

Histological evaluations were done by two expert histopathologists (AA and HU) who were familiar with the histological activity in UC and had no knowledge of the clinical findings. They discussed their evaluation and determined the histological activity by using the Riley Index. Six histological features of UC were factored: acute inflammatory cell infiltrate (polymorphonuclear cells in the lamina propria), crypt abscesses, mucin depletion, surface epithelial integrity, chronic inflammatory cell infiltrate (round cells in the lamina propria), and crypt architectural irregularities. Each feature was graded on a four-point scale from 0 to 3 (Table 3).

The outcomes of the AFI analysis using the RGB color model were compared with the endoscopic activity, endoscopic and histological features at the same sites. In addition, the ability of AFI to detect the microscopic inflammatory lesions which were extensively infiltrated by polymorphonuclear cells within MES-0 was evaluated.

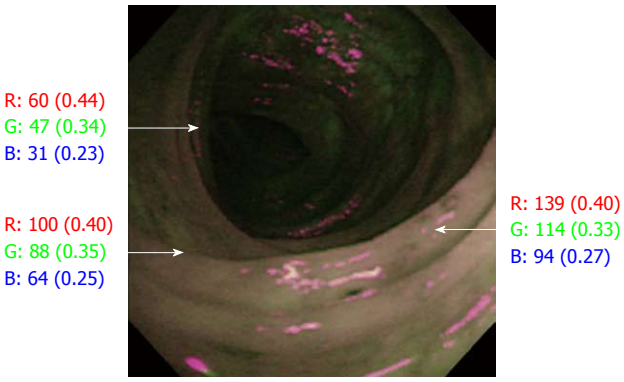

Figure 1 Endoscopic photograph by autofluorescence imaging showing the actual counts of red, green and blue colors based on the RGB additive model in different mucosal areas. The actual counts of red, green and blue colors varied depending on the brightness of the examined area, but the abundance of the actual counts, in parentheses, was approximately constant in the same picture.

Statistical analyses

The relative abundance of color components was expressed as the mean ± SD values. The relationships between endoscopic findings, histological evaluations, and the relative abundance of red, green and blue color components based on the RGB color model were determined by using the Spearman rank correlation coefficient (r). The Tukey-Kramer multiple comparison test was used for statistical analysis of the rate of color components based on the RGB color model among MES from 0 to 3. Before the Tukey-Kramer multiple comparison test, one-way analysis of variance (ANOVA) was performed. The Student's t-test (unpaired) was applied to compare the green color components based on the RGB color model between limited (combined grades 0 and 1) and extensive (combined grades 2 and 3) polymorphonuclear cell infiltration. Differences with P values < 0.05 were considered to be statistically significant.

RESULTS

The relationships between the RGB color components of AFI and WLI findings were evaluated in 572 endoscopic images from a total of 286 sites (Figure 2). Relative to the red (r = 0.52, P < 0.01) or blue (r = 0.56, P < 0.01) color component, the green color component (r = -0.62, P < 0.01) corresponded most often with sites of mucosal inflammation in different colonoscopy images (Figure 3A). However, there was an inverse relationship between the green color components and mucosal inflammation, so that the green color components of AFI diminished as the mucosal inflammation became more severe.

The average number of green color components for images classified into each MES subscore was 0.396 ± 0.043 for MES-0, 0.340 ± 0.035 for MES-1, 0.324 ± 0.034

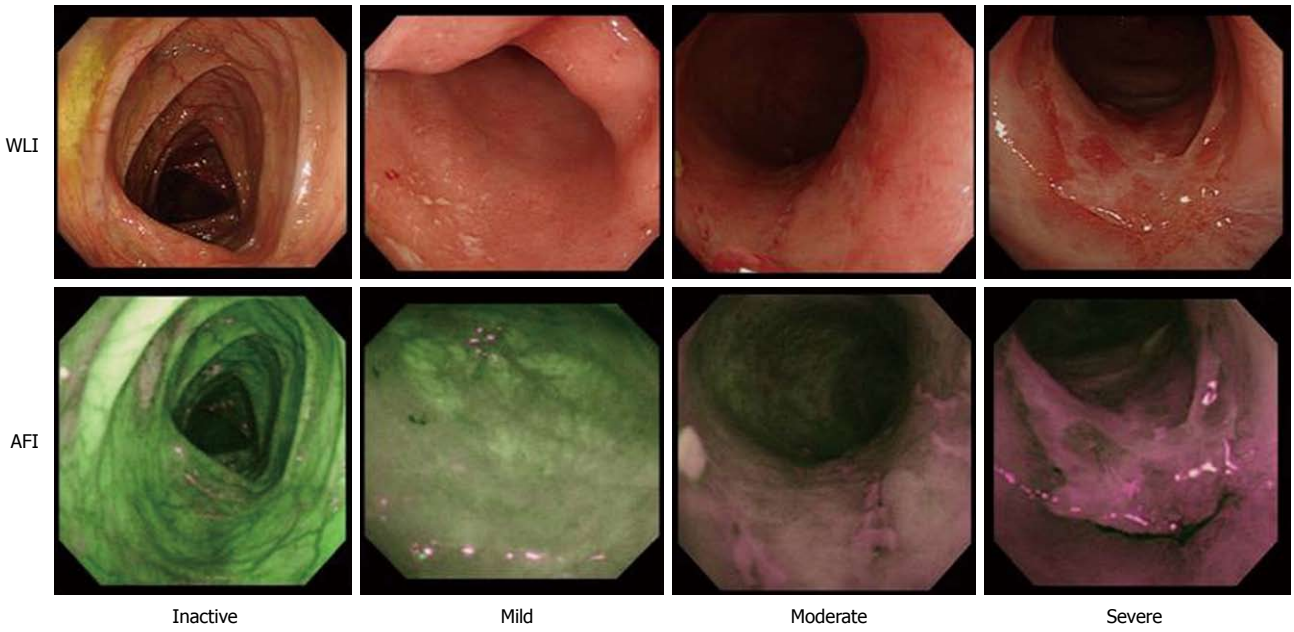

Figure 2 Representative endoscopic photographs of ulcerative colitis using white light imaging (upper row) and autofluorescence imaging (lower row) at the same sites according to the level of endoscopic ulcerative colitis activity (inactive, mild, moderate and severe). The color of the large intestinal mucosa on the autofluorescence imaging (AFI) changes by degrees, from green to grayish and magenta color. WLI: White light imaging.

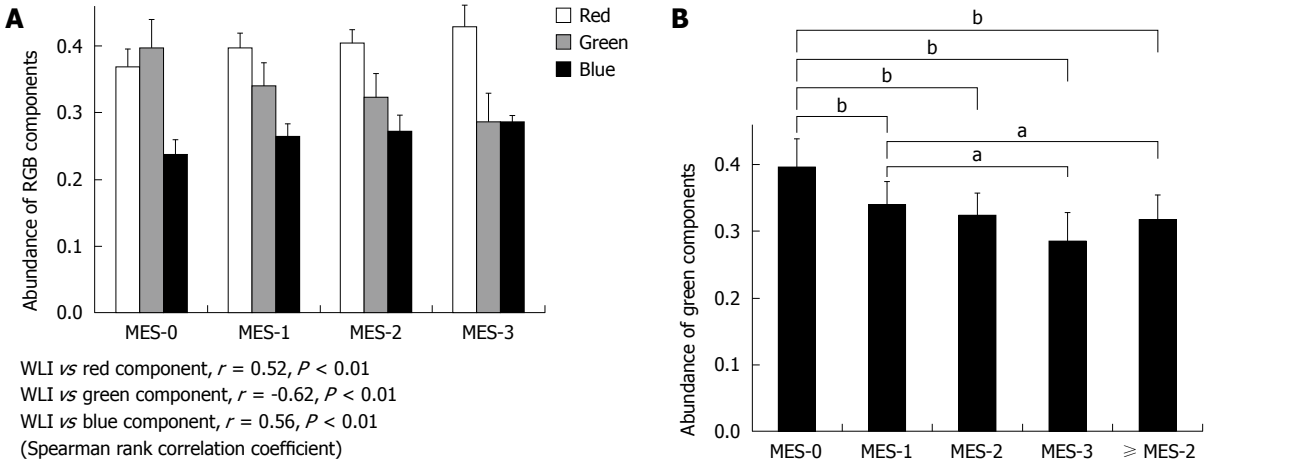

Figure 3 Comparison of overall mucosal inflammation on white light imaging and the abundance of RGB color components on autofluorescence imaging. A: The green color component corresponds most often with sites of mucosal inflammation in different colonoscopy images (according to Spearman rank correlation coefficient); B: There are significant differences in the green color component values between Mayo endoscopic subscore (MES)-0 and MES-1 ($^{b}P < 0.01$), between MES-0 and MES-2 ($^{b}P < 0.01$), between MES-0 and MES-3 ($^{b}P < 0.01$), between MES-1 and MES-3 ($^{a}P < 0.05$), between MES-0 and ⩾ MES-2 ($^{b}P < 0.01$) and between MES-1 and ⩾ MES-2 ($^{a}P < 0.05$), but not between MES-1 and MES-2 or between MES-2 and MES-3 (Tukey-Kramer multiple comparison test). WLI: White light imaging.

for MES-2, and 0.286 ± 0.043 for MES-3. There were significant differences in green color component values between MES-0 and MES-1 ($P < 0.01$), MES-0 and MES-2 ($P < 0.01$), MES-0 and MES-3 ($P < 0.01$), and MES-1 and MES-3 ($P < 0.05$), but not between MES-1 and MES-2, nor between MES-2 and MES-3. Likewise, there was a significant difference in green color component values between MES-1 and scores of ⩾ MES-2 (0.318 ± 0.037) as a combined group of MES-2 and MES-3 ($P < 0.01$) (Figure 3B). As the severity of the mucosal inflammation increased, the green color component of AFI decreased. However, the value of the color did not alter much between moderate and severe inflammation.

The relationships between the AFI green color component and WLI scores for the seven characteristic endoscopic features of UC are presented in Figure 4. The correlation coefficients for the AFI green color component with the scores of the seven characteristics were as follows: vascular pattern $r = -0.65$, $P < 0.01$; erythema $r = -0.55$, $P < 0.01$; edema $r = -0.62$, $P < 0.01$; granularity $r = -0.53$, $P < 0.01$; erosions $r = -0.45$, $P < 0.01$; ulcers $r = -0.23$, $P < 0.01$; and friability $r = -0.52$, $P < 0.01$. The AFI green color component was well correlated with the scores for vascular pattern and edema, but not with the

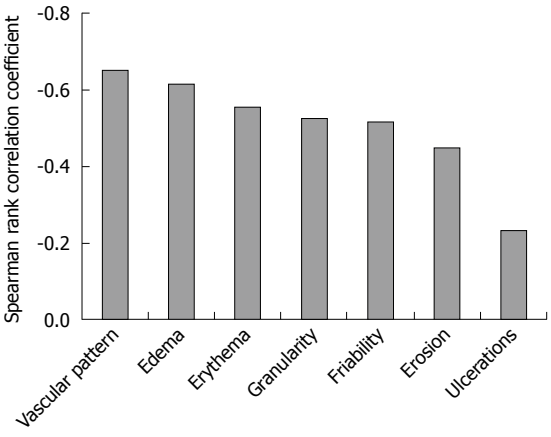

Figure 4 Comparison of seven endoscopic ulcerative colitis features on white light imaging and the green color component on autofluorescence imaging. The green color component on autofluorescence imaging is well correlated with vascular pattern (r = -0.65, P < 0.01) and edema (r = -0.62, P < 0.01) scores on WLI, but not with the ulcer score (r = -0.23, P < 0.01) (Spearman rank correlation coefficient). WLI: White light imaging.

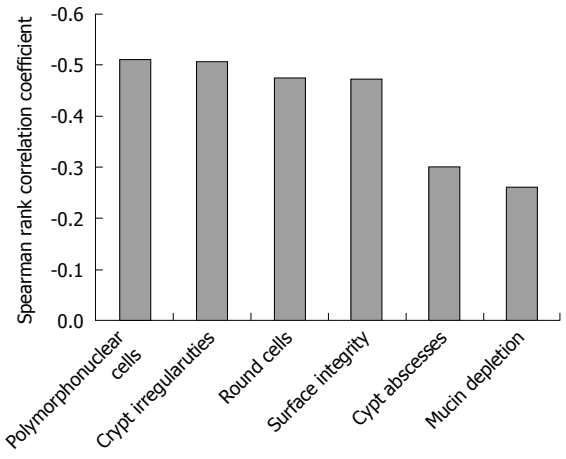

Figure 5 Comparison of histology scores, six histological characteristic ulcerative colitis features and the green color component on autofluorescence imaging. The autofluorescence imaging green color component is relatively well correlated with the scores for polymorphonuclear cells in the lamina propria (r = -0.51, P < 0.01) and crypt architectural irregularities (r = -0.51, P < 0.01), but not with the scores for crypt abscesses (r = -0.30, P < 0.01), and mucin depletion (r = -0.26, P < 0.01) based on Spearman rank correlation coefficient.

score for ulcers. It also showed relatively good correlation with the other endoscopic features of UC. The relationships between the AFI green color component and histology scores of six histological characteristic features of UC are shown in Figure 5. The correlation coefficients for the AFI green color component with the scores for the histological features were as follows: polymorphonuclear cells in the lamina propria r = -0.51, P < 0.01; crypt abscesses r = -0.30, P < 0.01; mucin depletion r = -0.26, P < 0.01; surface epithelial integrity r = -0.47, P < 0.01; round cells in the lamina propria r = -0.48, P < 0.01; and crypt architectural irregularities r = -0.51, P < 0.01. The AFI green color component was relatively well correlated with the scores for polymorphonuclear cells in the lamina propria

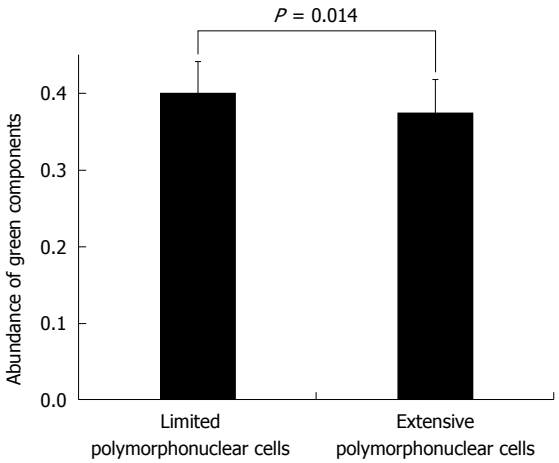

Figure 6 Comparison of limited (combined grades 0 and 1) and extensive (combined grades 2 and 3) polymorphonuclear cell infiltration of the green color components in Mayo endoscopic subscore-0. The mean number of green color components for images classified into the amount of polymorphonuclear cell infiltration in Mayo endoscopic subscore (MES)-0 is 0.399 ± 0.042 for limited and 0.375 ± 0.044 for extensive infiltration. There are significant differences in green color component values between limited and extensive polymorphonuclear cell infiltration in MES-0 (P = 0.014, by Student's t-test).

and with the crypt architectural irregularities, but not with the scores for crypt abscesses and mucin depletion.

The relationship between limited (combined grades 0 and 1) and extensive (combined grades 2 and 3) polymorphonuclear cell infiltration based on the green color components within MES-0 based on WLI are shown in Figure 6. The mean numbers of green color components for images classified according to the level of polymorphonuclear cell infiltration within MES-0 were 0.399 ± 0.042 and 0.375 ± 0.044 for limited and extensive infiltration, respectively. There were significant differences in green color component values between limited and extensive polymorphonuclear cell infiltration within MES-0 (P = 0.014). The abundance of green color components of AFI was also significantly different between microscopic inflammatory sites and histologically-quiescent UC lesions.

DISCUSSION

AFI has been used to highlight neoplastic tissue[10], minimal change in reflux esophagitis[11], the extent of chronic atrophic fundal gastritis[12], and Barrett's esophagus[13]. In all of these reports the lesions on the AFI are divided into two colors, such as green and magenta, green and pink, green and gray, green and purple, because investigators were concerned with the presence and the extent of the lesions, rather than with determining the severity of inflammation. In the present study, AFI pictures were scored according to the relative abundance of red, green and blue color components within each image based on an RGB additive model. Therefore, each component was expressed as a sequential variable number, and this facilitated evaluation of the severity of mucosal damage.

Further, the analysis was convenient to perform by using the Adobe® Photoshop® Elements® 5.0 software. Among the three color components, the green color component corresponded most often (and inversely) with sites of mucosal inflammation. Although fluorophores exist in both the mucosa and the submucosa, collagen in the submucosa emits strong green autofluorescence[14]. Our impression is that the presence of mucosal inflammation caused a high cell density and thickened the mucosal layer, making it difficult for the autofluorescence from the submucosa to penetrate the inflamed mucosa. Therefore, the green color component was reduced. The degree of the green color component might be valuable for assessing the extent of inflammation in the large intestine.

The overall mucosal inflammation in WLI, as assessed by MES, and the green color component in the AFI were well correlated. Specifically, there were significant differences in green color component values between MES-0 and MES-1, and between MES-1 and ⩾ MES-2, but not between MES-2 and MES-3. The degree of mucosal structural change around erosions and ulcers due to inflammation may differ only slightly between MES-2 and MES-3. Accordingly, the green color component of the AFI may not have been able to clearly distinguish moderate from severe inflammation.

Regarding the relationships between each of the seven characteristic endoscopic UC features on the WLI and the green color component of the AFI, vascular pattern and edema were well correlated with the green color component. These features are present from the early phase of UC, and decreased vascular pattern was particularly associated with MES-1, Baron score 1[15], modified Baron score 1[16], and Rachmilewitz index score 1[17]. This suggests that the green color component of the AFI is appropriate for detecting mucosal inflammation. In contrast, ulceration was not well correlated with the green color component of the AFI. The level of penetration of the green color component of the AFI from the submucosa through more than moderately inflamed mucosa might be constant regardless of the presence of ulcers. However, it was relatively simple to distinguish moderate from severe inflammation by using only WLI, based on the findings from spontaneous bleeding and multiple ulcers.

It has been reported that two measures of histological findings, such as architectural abnormalities including crypt architectural distortion and inflammatory features including large numbers of neutrophils, allow a distinction between inflammatory bowel disease and other causes of colorectal inflammation[18]. In our study, among six histological characteristic features of UC, polymorphonuclear cells in the lamina propria and crypt architectural irregularities were relatively well correlated with the green color component of the AFI. These two features are common in UC[19]. Polymorphonuclear cells in the lamina propria and crypt architectural irregularities are recognized in the active disease phase. The green color component of the AFI might reflect active mucosal inflammation. Other histological features of UC, especially mucin depletion and crypt abscesses, were not correlated with the green color component of the AFI. Although mucin depletion is a characteristic feature of UC, the degree of mucin depletion is not associated with the degree of inflammation. Crypt abscesses were observed in only about 7% of the biopsy specimens (26.2% of patients).

Although endoscopic remission was obtained after treatment, histological evidence of acute inflammatory cells often remained. The presence of microscopic inflammation contributed a 2- to 3-fold increase in the relapse rate[8]. The microscopic findings on inflammation were helpful in predicting relapse and for deciding on medical intervention. In this study, although the difference between microscopic inflammation and histological quiescence and the intensity of the green color components was relatively small, nevertheless the difference was significant. It is likely that microscopic inflammation was determined based on an evaluation of the green color component of the AFI.

In conclusion, the outcomes of the present investigation indicate that AFI is an appropriate new approach for detecting inflammatory regions and approximately estimating the severity of inflammation in UC patients, regardless of endoscopic experience. In particular, microscopic to moderate inflammation can be detected based on the green color component of the RGB additive color model. Further studies should strengthen our findings on the clinical relevance of AFI in patients with UC.

COMMENTS

Background

Endoscopic and histological findings define the degree of inflammatory activity in the clinical diagnosis of ulcerative colitis (UC). Although endoscopy is the most immediate method for assessing intestinal mucosal damage, the consistency of inter- and intra-observer findings and diagnoses have been reported to vary considerably. Substantial experience is necessary to accurately assess the disease activity in UC, particularly as microscopic inflammation cannot be detected by using conventional endoscopy.

Research frontiers

Autofluorescence imaging (AFI) videoendoscopy produces real-time pseudocolor images based on tissue autofluorescence emitted by excitation of endogenous tissue fluorophores. AFI has been used to highlight various lesions. There is no report on the change of hue related to AFI indicating extent and severity of UC activity.

Innovations and breakthroughs

AFI images were evaluated by analyzing the hue, and were scored relative to the abundance of red, green and blue color components of the RGB color model. As the green color components of AFI diminished, the mucosal inflammation was indicated to be more severe.

Applications

AFI appears to be an appropriate new approach and should be valuable for detecting inflammatory regions and approximately estimating the severity of inflammation in UC patients regardless of endoscopic experience, but based on the green color components of the RGB additive color model.

Terminology

AFI is a novel technique for detecting the autofluorescence emitted by the gastrointestinal tissues, mainly from collagen type-I in the submucosal layer, in real time. In an AFI mode, excitation light (395-475 nm) for inducing autofluorescence, together with green light (550 nm) and red light (610 nm) for obtaining reflection images, are generated by a light source equipped with a 300W xenon

arc lamp through a rotating filter. An excitation light cut filter is incorporated in the CCD for the AFI mode to allow only 490-625 nm light to reach the CCD.

Peer review

The present study demonstrates some technical progress when the AFI pictures were scored in a new way and the green color component correlated to the inflammatory changes on a significant level. However, the difference between mild inflammation and severe inflammation was small (about 10% in the abundance of green light components) and therefore our attitude to the results and conclusion should be cautious. As a preliminary and pioneering work this could be accepted for publication.

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BRIEF ARTICLE

Aspirin-induced small bowel injuries and the preventive effect of rebamipide

Kazuhiro Mizukami, Kazunari Murakami, Takashi Abe, Kunimitsu Inoue, Masahiro Uchida, Tadayoshi Okimoto, Masaaki Kodama, Toshio Fujioka

Kazuhiro Mizukami, Kazunari Murakami, Takashi Abe, Kunimitsu Inoue, Masahiro Uchida, Tadayoshi Okimoto, Masaaki Kodama, Toshio Fujioka, Department of General Medicine and Gastroenterology, Faculty of Medicine, Oita University, Yufu, Oita 879-5593, Japan
Author contributions: Mizukami K, Murakami K, and Fujioka T designed and performed research, analyzed data, and wrote the paper; Abe T, Inoue K, Uchida M, Okimoto T and Kodama M performed research and analyzed data.
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Correspondence to: Kazuhiro Mizukami, Dr., MD, Department of General Medicine and Gastroenterology, Faculty of Medicine, Oita University, 1-1, Idaigaoka, Hasama, Yufu, Oita 879-5593, Japan. mizkaz0809@oita-u.ac.jp
Telephone: +81-97-5866193 Fax: + 81-97-5866194
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Abstract

AIM: To evaluate the influence of taking low-dose aspirin for 4 wk on small intestinal complications and to examine the preventive effect of rebamipide.

METHODS: This study was conducted as a single-center, randomized, double-blind, cross-over, placebo-controlled study. Eleven healthy male subjects were enrolled. Each subject underwent video capsule endoscopy after 1 and 4 wk of taking aspirin and omeprazole, along with either rebamipide or placebo therapy. The primary endpoint was to evaluate small bowel damage in healthy subjects before and after taking low-dose aspirin for 4 wk.

RESULTS: The number of subjects with mucosal breaks (defined as multiple erosions and/or ulcers) were 1 at 1 wk and 1 at 4 wk on the jejunum, and 6 at 1 wk (P = 0.0061) and 7 at 4 wk on the ileum (P = 0.0019). Rebamipide significantly prevented mucosal breaks on the ileum compared with the placebo group (P = 0.0173 at 1 wk and P = 0.0266 at 4 wk).

CONCLUSION: Longer-term, low-dose aspirin administration induced damage in the small bowel. Rebamipide prevented this damage, and may be a candidate drug for treating aspirin-induced small bowel complications.

Key words: Healthy subjects; Low-dose aspirin; Small bowel injury; Capsule endoscopy; Rebamipide

Peer reviewer: Rami Eliakim, Professor, Department of Gastroenterology, Rambam Medical Center, Haifa 31096, Israel

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INTRODUCTION

Video capsule endoscopy (VCE)[1-2] is a practical technique that can be used to identify the causes and sites of obscure gastrointestinal bleeding. VCE allows for prospective investigation of small intestinal injuries, which frequently occur following the administration of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin. For example, Graham *et al*[3] used VCE and reported that small intestinal injury occurred in 71% of chronic NSAID users.

Low-dose aspirin is currently recommended for the secondary prevention of cardiovascular and cerebral diseases[4-6]. An observational registry reported that 70% to 80% of patients with a high risk of atherothrombosis

were receiving low-dose aspirin to prevent future vascular events[7]. Nonetheless, low-dose aspirin is not without risks. For instance, Lanas *et al*[8]reported that taking an anti-platelet agent induced lower, as well as upper, gastrointestinal (GI) events (16.9% and 15.5%, respectively). To date, there has been considerable interest in preventing upper gastrointestinal complications of NSAIDs; however, it has become clear that a strategy to prevent small bowel complications may also be needed[9].

It is not yet clear what duration of low-dose aspirin ingestion causes small bowel damage. Moreover, the frequency and severity of small bowel damage from taking low-dose aspirin is not yet known. Three recent reports investigating aspirin-induced small bowel damage were all short-term (1 to 2 wk) investigations[10-12]. These studies reported mild injuries (20% to 60%), such as erosion, in addition to more serious injuries (0 to 10%), such as ulcers[10-12]. The use of low-dose aspirin for cardiovascular prophylaxis is generally long-term, and it is clear that longer term observational studies are needed to examine damage to the small intestines.

The aims of this study were to investigate the frequency and type of small bowel damage associated with a 4 wk administration of low-dose aspirin in healthy subjects and to investigate the preventive effect of the cytoprotective agent, rebamipide, on aspirin-induced small bowel damage.

MATERIALS AND METHODS

The study protocol was approved by the Ethics Committees of Oita University, and written informed consent was obtained from all subjects. Eligible subjects were aged 20 to 65 years, who had taken no drugs during the one-month period prior to the start of this study and who had normal physical examinations. The exclusion criteria were as follows: (1) subjects who did not have a full length, small bowel VCE prior to the start of the study; (2) subjects with stenosis, tumors, ulcers, erosions, or bleeding in the small bowel; (3) subjects who had active GI disease or a history of ulcers, surgery, or bleeding; and (4) subjects who had used any medication, including NSAIDs or aspirin, within 4 wk of the start of the study.

Treatment protocol and post-treatment capsule endoscopy

This study was conducted using a cross-over design as shown in Figure 1. Medication groups A and B were defined as follows: group A: placebo plus aspirin (Bayer Pharmaceutical Co., Ltd., Tokyo, Japan) plus omeprazole (Sawai Pharmaceutical Co., Ltd., Osaka, Japan) for 4 wk; and group B: rebamipide (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) plus aspirin plus omeprazole for 4 wk (rebamipide 300 mg or placebo tid, aspirin 100 mg od, omeprazole 20 mg od). Omeprazole was used primarily for ethical reasons to avoid the effect of aspirin on the stomach.

Medications were administered for 4 wk. Then following a 4-wk washout period, the treatments were reversed for the two groups, and the medications were administered for a second 4-wk period. The washout period was designed according to a previous study by Niwa[13]. VCEs of the small bowel were performed five times: prior to the intervention, at 1 and 4 wk during the first period, and at 1 and 4 wk during the second treatment period.

Allocation and randomization were conducted by an independent pharmacologist of Yamanami Pharmacy who had no connection to our institution or the results of this study. The placebo was prepared by Yamanami Pharmacy.

Evaluation of small intestinal injuries

In this study, erosion and ulcer were defined according to Graham's report[3]. Red spots were defined as red areas without clear mucosal break. Erosions were defined as circumscribed areas of mucosal disruption denuded of villi with or without exudates or red color that involved a diameter equivalent to the valvulae conniventes. Ulcers were defined as erosions with a central area with exudates typically having a surrounding border of elevated mucosa, producing a target lesion or coral polyp appearance[3]. Typical cases of red spots and erosion are shown in Figures 2A and B, and an example of an ulcer is shown in Figure 2C.

Mucosal break was defined as two or more erosions and/or an ulcer. Red spots were scored, but were not considered as a significant injury, since they can be observed normally. The location of the injury was also scored in terms of the locations as proximal (jejunal) or distal (ileal) based on the transit time. The transit time from the pylorus to cecum was divided in half, and the first portion was arbitrarily defined as the jejunal section. Cases with erosion, multiple erosions, and ulcers were calculated. Multiple erosions were defined as more than 2 erosions in a subject. Subjects were only analyzed if the entire small bowel was observable by VCE.

Endpoints

The primary endpoint was to evaluate healthy subjects before and after 4 wk of low-dose aspirin-induced small bowel damage. The secondary endpoint was to evaluate the preventive effect of rebamipide.

Capsule endoscopy

We used the Olympus video capsule system (EndoCapsule, Olympus Ltd.; Tokyo, Japan) for this study. The capsule endoscopy procedure and the methodology for reviewing the images were conducted as previously described. All video images were analyzed twice by each skilled reviewer (KM, TA, and KI). These three investigators were instructed to mark any significant lesions under blinded conditions, and to evaluate lesions according to criteria for determination of the endpoints. If the results differed between the reviewers, then they consulted one

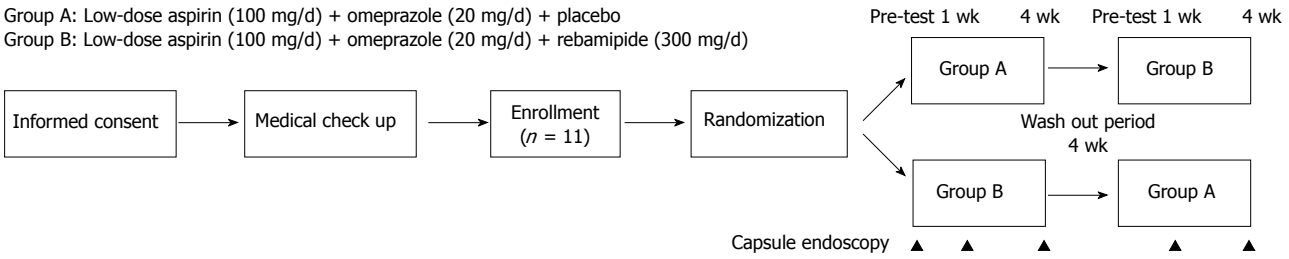

Figure 1 Study design.

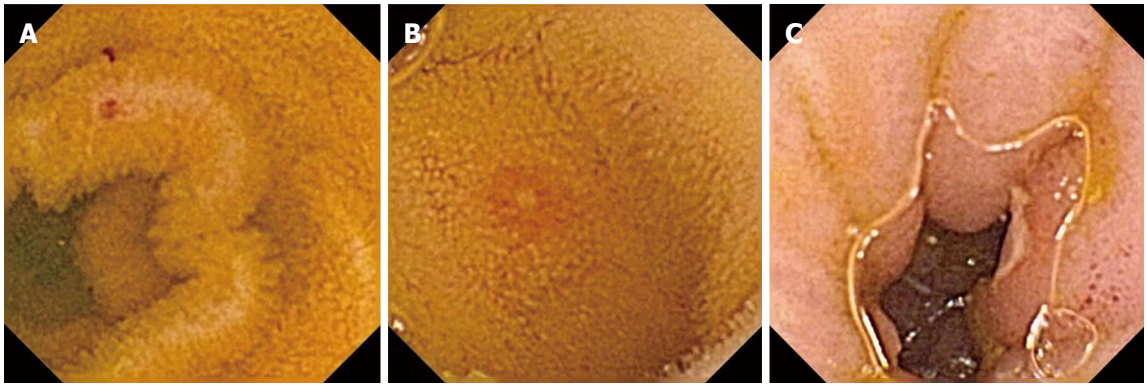

Figure 2 Typical small intestinal red spot, erosion and ulcer. A: Typical red spot; B: Typical erosion; C: Typical ulcer.

another to achieve a consensus. All images were saved for a final comprehensive analysis upon completion of all of the post-treatment capsule endoscopies.

Safety assessment

The subjects' symptoms were observed daily throughout the study periods, and the information was evaluated using a patient diary.

Statistical analysis

The primary endpoint was to evaluate the proportion of healthy subjects with small intestinal injury after 4 wk of low-dose aspirin therapy. The number of healthy subjects with small bowel mucosal breaks, ulcers, erosions, multiple erosions, and red spots were calculated and treated as parametric parameters. The small bowel area was divided into the jejunum and the ileum. The number of small bowel ulcers, erosions, and red spots were described as the mean ± SD. These values were analyzed at each measured point of the VCE in each group using Mann-Whitney's *U* test and compared with the evaluation prior to the aspirin administration.

The secondary endpoint was to compare the number of small intestinal injuries between the placebo and the rebamipide groups. The injuries were described as means ± SD and treated as nonparametric parameters. These injuries were analyzed at each measured point of the VCE in each group using Mann-Whitney's *U* test. In addition, the small intestinal injuries were compared between the placebo and rebamipide groups.

This study was a pilot study. Therefore, there were no reference data to calculate the sample size. A *P*-value < 0.05 was considered significant. All statistical analyses were performed using SAS version 8.2 (SAS Institute, Cary, NC, United States).

RESULTS

Eleven healthy subjects were enrolled in this study. The mean age of the subjects was 30 ± 6 years. The median age was 27 years, and the range was 24 to 43 years.

Influence of low-dose aspirin on small bowel mucosa in the placebo group

The number of subjects with multiple erosions, ulcers, and mucosal breaks of the small bowel are shown in Table 1. There were 8 subjects with red spots on the jejunum before the administration of aspirin, 10 subjects at 1 wk, and 10 at 4 wk. There were 8 subjects with red spots on the ileum before administration of aspirin, 11 subjects at 1 wk, and 11 at 4 wk. There were 2 subjects with small bowel erosion on the jejunum at 1 wk and 4 at 4 wk ($P = 0.0379$). There were 7 subjects with small bowel erosion on the ileum at 1 wk ($P = 0.0019$) and 9 at 4 wk ($P < 0.0001$).

The numbers of small bowel injuries before and after aspirin treatment are shown in Figure 3. There were 6.3 ± 6.9 small intestinal red spots at 1 wk and 9.6 ± 6.6 at 4 wk on the jejunum, and 68.0 ± 133.6 at 1 wk ($P = 0.0010$) and 48.4 ± 67.7 at 4 wk ($P = 0.0010$) on the ileum. There were 0.2 ± 0.6 small bowel erosions at 1 wk and 0.4 ± 0.6 at 4 wk on the jejunum, and 2.1 ± 2.6 at 1 wk ($P = 0.0156$)

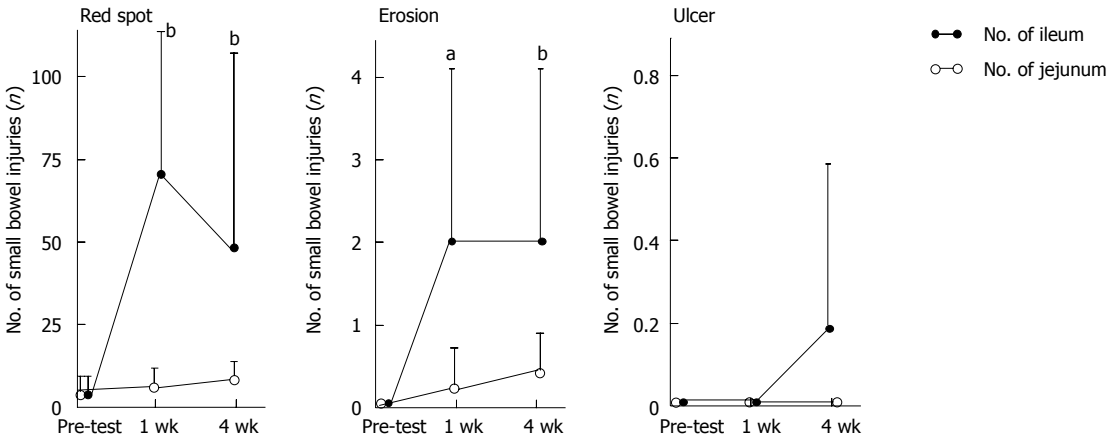

Figure 3 Low-dose aspirin-related small bowel injuries in the placebo group. The open circle indicates the number of low-dose aspirin-related small bowel injuries in the jejunum, and the closed circle indicates the number of injuries in the ileum at 4 wk. The data are represented as the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$.

Table 1 No. of healthy subjects presenting small bowel injuries following a 4 wk regimen of low dose aspirin in the placebo group and evaluation of preventive efficacy (n = 11)

	Jejunum			Ileum		
	Pre-test	1 wk	4 wk	Pre-test	1 wk	4 wk
Placebo group						
Multiple erosion (*n*)	0	1	1	0	6[b]	4[a]
Ulcer (n)	0	0	0	0	0	3
Mucosal break (*n*)	0	1	1	0	6[b]	7[b]
Rebamipide group						
Mucosal break (*n*)	0	0	1	0	1[c]	2[c]

Multiple erosion (*n*): No. of subjects with multiple erosions in jejunum or ileum; Ulcer (*n*): No. of subjects with ulcer in jejunum or ileum; Mucosal break (*n*): No. of subjects with multiple erosion and/or ulcer in jejunum or ileum. Statistical analysis was performed by Mann Whitney's *U* test, compared to before administration *vs* 1 wk and 4 wk. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.05$ compared with placebo and rebamipide group.

and 2.0 ± 2.5 at 4 wk ($P = 0.0039$) on the ileum. There were no small intestinal ulcers at 1 wk and at 4 wk on the jejunum, and there were no small intestinal ulcers at 1 wk and 0.2 ± 0.4 ulcers at 4 wk on the ileum.

Evaluation of the preventive effect of rebamipide

The preventive effect of rebamipide is shown in Table 1. There were no subjects with small bowel mucosal breaks on the jejunum at 1 wk and 1 subject at 4 wk, and there was 1 small bowel mucosal break on the ileum at 1 wk and 2 at 4 wk. Rebamipide significantly prevented small bowel mucosal breaks on the ileum compared with the placebo group ($P = 0.0173$ at 1 wk and $P = 0.0266$ at 4 wk). There were 15.7 ± 8.5 red spots on the ileum at 4 wk in the rebamipide group, which was significantly fewer than in the placebo group ($P = 0.0354$). There were 0.6 ± 1.2 erosions on the ileum at 4 wk, which was significantly fewer that in the placebo group ($P = 0.0362$).

Safety assessment

We examined symptoms daily for all subjects throughout the study period. Three subjects with ulcers were observed in the placebo group. Two of these subjects were symptomatic, but not anemic. The symptoms of these two subjects diminished 1 month after the study ended. One subject with an ulcer was observed in the rebamipide group, but the subject was neither symptomatic nor anemic.

DISCUSSION

In our study, we observed that the administration of low-dose aspirin induced small bowel red spots and erosions in healthy subjects in the early phase (1 wk). Red spots were observed in all subjects with a mean of 68.0 ± 133.6 at 1 wk and 48.4 ± 67.7 at 4 wk. There were 7 subjects with erosions on the ileum at 1 wk and 9 at 4 wk in the placebo group. These small bowel injuries were induced in the early phase and maintained while taking low-dose aspirin. On the other hand, small intestinal ulcers were observed in 3 subjects in the late phase (4 wk), although no ulcers were observed at 1 wk. These results indicate that serious small bowel injury, such as ulcers, may be induced by longer-term administration of low-dose aspirin. In addition, a very large ulcer was observed in one case at 4 wk (Figure 4), although no ulcer was observed in this subject at 1 wk. This may demonstrate a risk of longer-term low-dose aspirin ingestion. Patients taking low-dose aspirin should be given periodic management. In this study, small bowel bleeding was not observed; however, some patients may present small bowel bleeding due to the use of low-dose aspirin during treatment periods longer than 4 wk. The clinical implications of small bowel mucosal injuries are not yet clear. However, bleeding from mucosal injury can be fatal because aspirin is an anti-platelet agent. In this study, multiple erosions were also evaluated. They resulted in a more serious condition than a single erosion.

Our results indicate that most small bowel injuries occurred in the ileum rather than the jejunum area. There were 7 cases with a mucosal break in the ileum and 1 in the jejunum at 4 wk in the placebo group. Bjarnason *et al*[14] demonstrated that small bowel damage depended on various factors, such as microvascular aspects, neutrophil recruitment, mucosal prostaglandins,

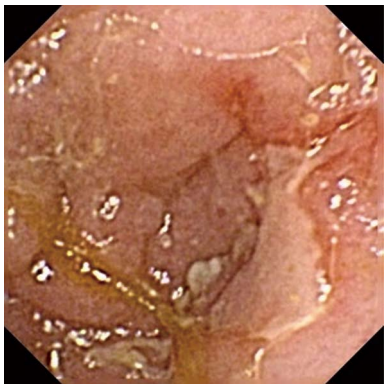

Figure 4 Case with a large ulcer of the small intestine induced by low-dose aspirin. A large ulcer was observed in the small intestine as a result of 4 wk of low-dose aspirin ingestion. The ulcer occupied one-third of the interior of the small intestine.

decreased blood flow, increased permeability, and bacteria. The number and variation of bacteria in the ileum is reported to be greater than in the jejunum[15]. The results of this study may be a reflection of the different bacterial environment in the ileum compared with the jejunum.

We conducted a comparative study with placebo and rebamipide to observe whether rebamipide could prevent aspirin-induced small bowel injuries. Niwa *et al*[13] reported that rebamipide had a preventive effect on diclofenac-induced small-bowel injury compared to the placebo. The preventive ratio of diclofenac-induced small bowel mucosal breaks was 60% in the placebo group and 20% in the rebamipide group, and this difference was statistically significant. Moreover, Fujimori *et al*[16] demonstrated that the prostaglandin analogue misoprostol prevented diclofenac-induced small-intestinal complications. These two reports indicate the importance of prostaglandins.

Rebamipide {2-(4-chlorobenzoylamino)-3-[2-(1H)-quinolinon-4-yl]-propionic acid} is a cytoprotective antiulcer drug that stimulates the production of endogenous prostaglandins[17]. Here, we observed that rebamipide significantly prevented small bowel mucosal breaks in the ileum area compared to the placebo group at both 1 wk and 4 wk. There were 3 subjects with ulcers in the ileum area at 4 wk in the placebo group, compared with 1 subject with an ulcer at 4 wk in the rebamipide group. The administration of rebamipide showed potential in preventing the incidence of ulcers; however, rebamipide does not have an anti-bacterial effect. The action of rebamipide may be explained by Bjarnason's hypothesis[14]. Previous reports have indicated that rebamipide induces the production of intracellular prostaglandins[14], improves blood flow[18], suppresses increases in permeability[19], scavenges free radicals[20], and has an anti-inflammatory action[21]. Presently, there is no drug to prevent aspirin-induced small bowel complications or treat patients with these complications, so it is essential to research drugs with these properties in the future.

A limitation of this study is that the study size was small. We did not use a large number of patients, since this was a preliminary study. In addition, since the small bowel is a long organ, it will be necessary to further investigate the appropriate dosage for this candidate drug.

In conclusion, the use of long-term low-dose aspirin induced small bowel damage. Rebamipide prevented this damage, and this candidate drug may be suitable for preventing aspirin-induced small bowel complications.

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COMMENTS

Background

Low-dose aspirin is currently recommended for the secondary prevention of cardiovascular and cerebral diseases. Recently, aspirin-induced small bowel complications have become the focus of investigations around the world.

Research frontiers

Few studies have observed healthy subjects with low-dose aspirin-induced, small bowel injuries using capsule endoscopy. These results demonstrated the prevalence of slight and low frequency small bowel injuries. However, these studies examined the effects of low-dose aspirin ingestion in the very short-term (1 or 2 wk). Here, we investigated the influence of small bowel damage following ingestion of low-dose aspirin for a longer period of 4 wk.

Innovations and breakthroughs

In this study, the ingestion of low-dose aspirin for 4 wk induced small bowel ulcers. In one case, a huge ulcer developed at 4 wk, although no ulcer was observed at 1 wk. Although it is common for patients to take low-dose aspirin for more than 4 wk, this duration of ingestion may be the limit in healthy subjects. Moreover, the results of this study demonstrate the differences in small bowel injuries after ingesting low-dose aspirin for 1 wk or 4 wk. Taking rebamipide was found to prevent aspirin-induced small bowel injuries.

Applications

The results of this study indicate that long-term use of low-dose aspirin can cause small intestinal injuries. Rebamipride could prevent this damage.

Terminology

Rebamipide is a cytoprotective antiulcer drug that stimulates the production of endogenous prostaglandins. Its actions include scavenging free radicals, elevating blood flow, and suppressing permeability. In this study, capsule endoscopy was performed using the Olympus video capsule system (EndoCapsule, Olympus Ltd.; Tokyo, Japan).

Peer review

This study focused on the influence of low-dose aspirin ingestion for 4 wk on small bowel damage in healthy subjects. The study was designed as a randomized, placebo-controlled, double-blind, cross-over study using video capsule endoscopy. The long-term (4 wk) use of low-dose aspirin induced small bowel damage. Rebamipide prevented this damage, and it may be a candidate drug for preventing aspirin-induced small bowel complications.

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BRIEF ARTICLE

Superiority of metastatic lymph node ratio to the 7th edition UICC N staging in gastric cancer

Long-Bin Xiao, Jian-Xing Yu, Wen-Hui Wu, Feng-Feng Xu, Shi-Bin Yang

Long-Bin Xiao, Jian-Xing Yu, Wen-Hui Wu, Feng-Feng Xu, Shi-Bin Yang, Department of Gastrointestinal-pancreatic Surgery, the First Affiliated Hospital, Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China
Author contributions: Xiao LB and Yu JX contributed equally to this work; Yu JX and Wu WH performed the majority of data collection and wrote the manuscript; Xu FF and Yang SB performed the statistical analysis; Xiao LB designed the study and provided financial support for this work.
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Correspondence to: Long-Bin Xiao, MD, Department of Gastrointestinal-pancreatic Surgery, the First Affiliated Hospital, Sun Yat-Sen University, 58 Zhongshan Road Ⅱ, Guangzhou 510080, Guangdong Province, China. longbinxiao@yahoo.cn
Telephone: +86-20-82379585 Fax: +86-20-82379495
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Abstract

AIM: To compare and evaluate the appropriate prognostic indicators of lymph node basic staging in gastric cancer patients who underwent radical resection.

METHODS: A total of 1042 gastric cancer patients who underwent radical resection and D2 lymphadenectomy were staged using the 6th and 7th edition International Union Against Cancer (UICC) N staging methods and the metastatic lymph node ratio (MLNR) staging. Homogeneity, discriminatory ability, and gradient monotonicity of the various staging methods were compared using linear trend χ^2, likelihood ratio χ^2 statistics, and Akaike information criterion (AIC) calculations. The area under the curve (AUC) was calculated to compare the predictive ability of the aforementioned three staging methods.

RESULTS: Optimal cut-points of the MLNR were calculated as MLNR0 (0), MLNR1 (0.01-0.30), MLNR2 (0.31-0.50), and MLNR3 (0.51-1.00). In univariate, multivariate, and stratified analyses, MLNR staging was superior to the 6th and 7th edition UICC N staging methods. MLNR staging had a higher AUC, higher linear trend and likelihood ratio χ^2 scores and lower AIC values than the other two staging methods.

CONCLUSION: MLNR staging predicts survival after gastric cancer more precisely than the 6th and 7th edition UICC N classifications and should be considered as an alternative to current pathological N staging.

Key words: Gastric cancer; Metastatic lymph node ratio; Prognosis

Peer reviewer: Lygia Stewart, MD, Professor of Clinical Surgery, University of California San Francisco, 4150 Clement Street, San Francisco, CA 94121, United States

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INTRODUCTION

In recent years, more cases of gastric cancer have been diagnosed in China than in any other country[1]. Accurate prognosis prediction for gastric cancer patients enables doctors to determine the patients' expected clinical courses and to have more information when deciding whether to use adjuvant therapy and when comparing the therapeutic effects of different treatment modalities. A widely used classification proposed by the International

Union Against Cancer (UICC), the tumor-node-metastasis (TNM) system, combines the most powerful and reliable factors for analyzing tumor status[2,3]. Lymph node metastasis is one of the most important gastric cancer prognostic factors[4]. The identified number of involved lymph nodes depends on the number of lymph nodes removed and examined, which in turn depends on the surgical and pathologic procedures. Although TNM classification is a convenient and reproducible method for precise staging, it demands the examination of at least 15 lymph nodes. If the number of dissected and examined lymph nodes is small, downmigration of N stage may occur, and conversely, if the number is large, upmigration of N stage may occur, which is also referred to as stage migration in some references[5-10]. To improve prognosis prediction, the number of positive lymph nodes should be considered in the context of the number of nodes examined. The metastatic lymph node ratio (MLNR), defined as the number of positive lymph nodes divided by the number of lymph nodes retrieved, has been proposed as an alternative to classification systems that assess the absolute number of positive lymph nodes, such as the UICC (2002, 6th edition) or Japanese Gastric Cancer Association (JGCA) (1998, 2nd English edition) staging systems[11-16].

This year, gastric cancer lymph node metastasis staging was changed in both the UICC 7th edition and the JGCA 14th edition staging systems in that it now depends solely on the number of metastatic nodes found[3,17]. In the new UICC and JGCA systems, patients with one to two positive lymph nodes are classified as N1, patients with three to six positive lymph nodes are classified as N2, and patients with seven or more positive lymph nodes are classified as N3. Some authors have demonstrated that the 7th edition UICC staging system is superior to the 6th edition based on its homogeneity, discriminatory ability and prognostic value[18-20].

However, to date there has been no formal study that focused on comparing the prognostic significance of the MLNR with that of the 7th edition UICC N staging system. In the present article, we investigate whether patients with gastric cancer can be classified into meaningful risk categories based on MLNR by comparing this staging system with the 7th edition UICC N staging system.

MATERIALS AND METHODS

Patients

Between January 1996 and December 2007, 1042 patients with histologically diagnosed gastric cancer underwent surgery at the Department of Gastrointestinal-pancreatic Surgery, First Affiliated Hospital, Sun Yat-Sen University, China. The postoperative pathological results included tumor size, histological type, margin, adjacent tissues and neighboring organs, lymphatic/venous invasion, retrieved lymph nodes, metastatic lymph nodes, and pTNM staging. The inclusion criteria of the study were as follows: (1) gastric adenocarcinoma identified by histo-pathological examination; (2) histologically confirmed R0 resection, which was defined as no macroscopic or microscopic residual tumor; and (3) availability of complete follow-up data. Patients with distant metastases, a history of familial malignancy or other synchronous malignancy (such as gastrointestinal stromal tumor, esophageal cancer, colorectal cancer, *etc.*), or carcinoma of the gastric stump after gastric resection for benign disease or who died in the perioperative period were excluded from the study.

D2 lymphadenectomy was performed by experienced surgeons following the JGCA guidelines[21]. A total of 15 313 lymph nodes were retrieved, with a mean of 14.70 ± 10.25 lymph nodes per patient (25.14 ± 9.28 for patients with > 15 lymph nodes retrieved and 8.58 ± 3.87 for patients with ⩽ 15 lymph nodes retrieved) and a range from 3 to 66. The mean number of lymph nodes with evidence of metastasis was 6.40 ± 6.90 per patient (9.78 ± 9.42 for patients with > 15 lymph nodes retrieved and 4.15 ± 2.83 for patients with ⩽ 15 lymph nodes retrieved), with a range from 1 to 70. Lymph node involvement was classified according to the 7th edition UICC (2010) N staging system (N0: no metastasis; N1: 1-2 metastatic lymph nodes; N2: 3-6 metastatic lymph nodes; N3: ⩾ 7 metastatic lymph nodes) and 6th edition UICC (2002) N staging system (N0: no metastasis; N1: 1-6 metastatic lymph nodes; N2: 7-15 metastatic lymph nodes; N3: ⩾ 16 metastatic lymph nodes). All nodal material was separately dissected from the specimen by a surgeon at the end of the procedure. Our study does not include stage Ⅳ patients, graded according to the UICC 7th edition staging system, because all of the patients enrolled underwent radical resection and had no distant metastasis.

Follow-up

Postoperative follow-up at our outpatient department included clinical and laboratory examinations every 3 mo for the first 2 years, every 6 mo during the third to fifth years, and annually thereafter until at least 5 years after the operation or until the patient died, whichever came first. Overall patient survival, defined as the time from operation to death or final follow-up, was used as a measure of prognosis. The median follow-up for the entire cohort was 56 mo (range 3-178 mo).

Statistical analysis

To determine the appropriate MLNR cut-points in the entire cohort, our analysis for the best cut-points was conducted as follows: In the first step, we evaluated the prognostic value of the MLNR, adjusting for other clinicopathological covariates that are significantly associated with gastric cancer mortality. Second, patients having no involved lymph nodes (MLNR = 0) were assigned to one group because it has been well documented that their prognosis significantly differs from patients with metastatic lymph nodes[12,15,22,23]. After ascertaining that the MLNR was significantly associated with gastric cancer mortality, we determined two additional appropriate cut-points for categorizing the MLNR to make our cut-

points comparable with those for UICC N staging. For this, we recomputed the likelihood associated with all possible pairs of MLNR cutoffs ranging from 0.05 to 0.95 at intervals of 0.05. In our study, the two alternative cut-points for the MLNR were 0.30 and 0.50. Martingale residual analysis was also used to examine the function form of the MLNR, and our cut-points (0, 0.30 and 0.50) were found to be consistent. After extensive evaluations of our data, no other sets of cut-points performed better than those already described. Thus, four subgroups of the MLNR classification (MLNR0, 0%; MLNR1, 1%-30%; MLNR2, 31%-50%; MLNR3, 51%-100%) were used in our study.

To directly compare the 6th and 7th edition UICC N staging systems with the present MLNR staging system, we took advantage of two statistical methods. One method considers the homogeneity, discriminatory ability and monotonicity of the gradient test. Homogeneity was measured with the likelihood ratio χ^2 test related to the Cox regression model. The discriminatory ability and monotonicity of the gradient were measured with the linear trend χ^2 test. The likelihood ratio χ^2 test was used to assess homogeneity within each classification system and to estimate the gradient monotonicity. Additionally, the Akaike information criterion (AIC) value within a Cox proportional hazard regression model was used to measure the discriminatory ability of each system[24]. The AIC statistic was defined by AIC = -2 log maximum likelihood + 2 × the number of parameters in the model. A smaller AIC value indicates that the model is better at predicting outcome. The other method involves receiver operating characteristic (ROC) curves. ROC curves and the areas under the curves (AUC) were calculated for each of the aforementioned three N staging systems to assess the accuracy of their predictive ability. Differences between the AUC were tested for statistical significance based on the estimated areas and their standard errors[25].

The 5-year survival rate was calculated using the Kaplan-Meier method. The log-rank test was used to make statistical comparisons of different factors. Pearson correlations were examined with a two-tailed test. In multivariate analysis, forward stepwise regression analysis was performed with a Cox proportional hazards model. A P value of $\leqslant 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Of the 1042 patients, 708 were male (67.9%) and 334 were female (32.1%). The mean patient age was 57.4 ± 11.5 years (range 20-79 years). The overall 5-year survival rate for all patients was 47.5%, and 474 patients were alive when our follow-up was complete.

Univariate and multivariate survival analysis

After univariate analysis of the 1042 patients who underwent radical resection, ten factors were found to have statistically significant associations with overall survival (OS). They were: age, tumor location, tumor size, histological grade, lymphatic/venous invasion, pT, 6th edition UICC pN, 7th edition UICC pN, MLNR, and the number of retrieved lymph nodes (Table 1). We summarize the postoperative survival results as follows: (1) patients who were older had significantly shorter OS than those who were younger [hazard ratio (HR) = 1.019, $P < 0.001$]; (2) patients whose primary tumor was located in the distal third of the stomach had significantly longer OS than those whose primary tumor was located elsewhere in the stomach (HR = 0.735, $P < 0.001$); (3) patients with a larger primary tumor had significantly shorter OS than those with a smaller primary tumor (HR = 1.147, $P < 0.001$); (4) patients with poorly differentiated or undifferentiated adenocarcinoma had significantly shorter OS than those with well or moderately differentiated adenocarcinoma (HR = 1.254, $P < 0.001$); (5) patients with tumor lymphatic/venous invasion had significantly shorter OS than those without lymphatic/venous invasion (HR = 2.685, $P < 0.001$); (6) the deeper the primary tumor invasion, the shorter the OS of the gastric cancer patients (HR = 1.852, $P < 0.001$); (7) the higher the metastatic lymph node counts of the 6th edition UICC N stage, the shorter the OS of the gastric cancer patients (HR = 1.571, $P < 0.001$); (8) the higher the metastatic lymph node counts of the 7th edition UICC N stage, the shorter the OS of the gastric cancer patients (HR = 1.604, $P < 0.001$); (9) the higher the MLNR stage, the shorter the OS of the gastric cancer patients (HR = 1.776, $P < 0.001$); and (10) patients who had more than 15 lymph nodes retrieved had significantly longer OS than those who had $\leqslant$ 15 lymph nodes retrieved (HR = 0.616, $P < 0.001$). All of the aforementioned 10 variables were included in a multivariate Cox proportional hazards model (forward stepwise procedure) to adjust for the effects of covariates (Table 2). In that model, we demonstrated that age, tumor location, tumor size, histological grade, lymphatic/venous invasion, pT, the 7th edition UICC N staging, MLNR, and the number of retrieved lymph nodes were independent prognostic factors, while the 6th edition UICC N staging was excluded.

The survival curves developed according to the 6th and 7th edition UICC N staging systems and the MLNR staging system are shown in Figure 1. For all three staging systems, the Kaplan-Meier plot had good discriminatory ability in each group except in N2 and N3 of the 6th edition UICC N staging (Figure 1). The 5-year survival rates of N0, N1, N2, and N3 patients in the 6th edition UICC N staging were 71.1%, 43.3%, 21.4%, and 25.1%, respectively ($P < 0.001$, $P < 0.001$ and $P = 0.143$, respectively). The 5-year survival rates of N0, N1, N2, and N3 patients in the 7th edition UICC N staging were 71.1%, 50.7%, 37.5%, and 22.2%, respectively ($P < 0.001$, $P = 0.003$ and $P = 0.001$, respectively). The 5-year survival rates of MLNR0, MLNR1, MLNR2, and MLNR3 patients were 71.1%, 59.0%, 32.7% and 16.0%, respectively ($P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively).

Table 1 Univariate analysis of various clinicopathologic factors in 1042 cases of gastric cancer

Variable	*n* (%)	5-yr survival rate (%)	Log rank χ^2 value	Hazard ratio	*P* value
Gender			0.433	1.060	0.511
Male	708 (67.9)	48.6			
Female	334 (32.1)	45.1			
Age (continuous)	1042 (100)	47.5	124.704	1.019	< 0.001
Tumor location			78.529	0.735	< 0.001
Proximal	579 (55.6)	39.0			
Distal	418 (40.1)	62.0			
Two-thirds or more	45 (4.3)	17.5			
Tumor size (continuous)		47.5	124.704	1.147	< 0.001
Histological grade			18.407	1.254	< 0.001
Well/moderately differentiated adenocarcinoma	388 (37.2)	54.8			
Poorly differentiated adenocarcinoma	448 (43.0)	44.7			
Undifferentiated adenocarcinoma/signet-ring cell carcinoma/mucinous adenocarcinoma	206 (19.8)	39.8			
Lymphatic/venous invasion			65.905	2.685	< 0.001
No	954 (91.6)	50.0			
Yes	88 (8.4)	20.4			
Depth of invasion (7th edition)			172.456	1.852	< 0.001
T1	81 (7.8)	90.7			
T2	120 (11.5)	74.3			
T3	195 (18.7)	57.5			
T4a	538 (51.6)	35.9			
T4b	108 (10.4)	22.1			
The 7th edition UICC N			168.281	1.604	< 0.001
N0	332 (31.9)	71.1			
N1	211 (20.2)	50.7			
N2	268 (25.7)	37.5			
N3	231 (22.2)	22.2			
The 6th edition UICC N			160.982	1.571	< 0.001
N0	332 (31.9)	71.1			
N1	479 (46.0)	43.3			
N2	172 (16.5)	21.4			
N3	59 (5.7)	25.1			
Metastatic lymph node ratio			281.341	1.776	< 0.001
MLNR0	332 (31.9)	71.1			
MLNR1	277 (26.6)	59.0			
MLNR2	154 (14.8)	32.7			
MLNR3	279 (26.8)	16.0			
Retrieved lymph nodes			67.098	0.616	< 0.001
≤ 15	657 (63.1)	58.0			
> 15	385 (36.9)	68.4			

Table 2 Multivariate survival analysis results

Variables	Wald	*P* value	HR	95% CI
Age (continuous)	23.741	< 0.001	1.020	1.012-1.028
Tumor location	8.825	0.003	0.794	0.682-0.925
Tumor size (continuous)	29.678	< 0.001	1.085	1.054-1.118
Histological grade	11.542	0.001	1.222	1.089-1.372
Lymphatic/venous invasion	30.629	< 0.001	2.063	1.596-2.666
UICC 7th T	43.652	< 0.001	1.434	1.289-1.596
UICC 7th N	5.806	0.016	1.218	1.037-1.430
MLNR	14.693	< 0.001	1.330	1.149-1.538
Retrieved lymph nodes	29.666	< 0.001	0.548	0.441-0.680

CI: Confidence interval; UICC: International Union Against Cancer; HR: Hazard ratio; T: Tumor; N: Node; MLNR: Metastatic lymph node ratio.

We also investigated the impact of the number of lymph nodes retrieved on OS rates according to different N staging systems. In the 6th edition UICC N staging, the 5-year survival rate was significantly higher for patients with N0 compared with N1 and N2 in the ≤ 15 lymph nodes retrieved group, in which no patient was classified as N3 ($P < 0.001$ and $P < 0.001$, respectively). The Kaplan-Meier plot discriminated well between each N staging in the > 15 lymph nodes retrieved group ($P = 0.008$ and $P < 0.001$, respectively), except for N2 *vs* N3 ($P = 0.720$). As for the 7th edition UICC N staging, the Kaplan-Meier plot discriminated well between each N staging in both the ≤ 15 and > 15 lymph nodes retrieved groups, except for N0 *vs* N1 and N1 *vs* N2 ($P = 0.070$ and $P = 0.433$, respectively) in the > 15 lymph nodes retrieved group. When we investigated the MLNR in the ≤ 15 and > 15 lymph nodes retrieved groups, the Kaplan-Meier plot showed that the 5-year survival rate was significantly different for each MLNR stage. In the ≤ 15 lymph nodes retrieved group, the 5-year OS was 66.2%, 49.9%, 29.9%, and 11.2% for MLNR 0, 1, 2, and 3 ($P = 0.001$, $P < 0.001$ and $P < 0.001$, respectively). In the > 15

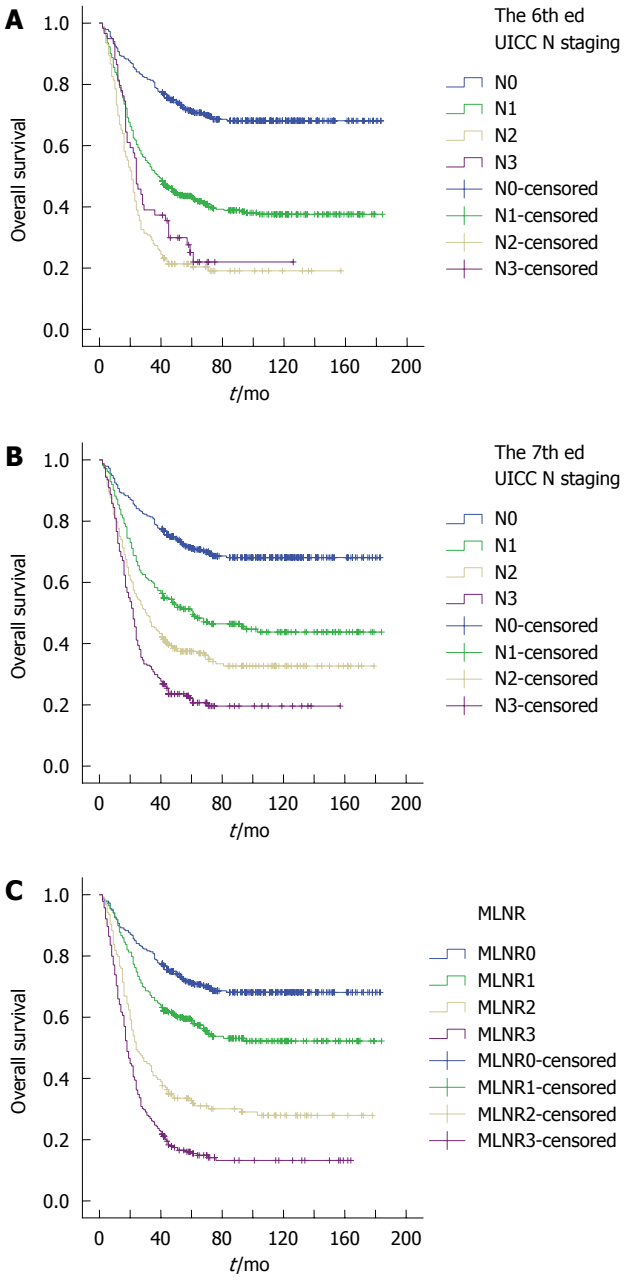

Figure 1 Impact of 6th and 7th International Union Against Cancer N staging systems (A and B) and metastatic lymph node ratio staging (C) on overall survival of gastric cancer patients who underwent radical resection. A: The 6th International Union Against Cancer (UICC) N staging: N0 *vs* N1, $P < 0.001$; N1 *vs* N2, $P < 0.001$; N2 *vs* N3, $P = 0.143$; B: The 7th UICC N staging: N0 *vs* N1, $P < 0.001$; N1 *vs* N2, $P = 0.003$; N2 *vs* N3, $P = 0.001$; C: Metastatic lymph node ratio (MLNR) staging: MLNR0 *vs* MLNR1, $P < 0.001$; MLNR1 *vs* MLNR2, $P < 0.001$; MLNR2 *vs* MLNR3, $P < 0.001$.

lymph nodes retrieved group, the 5-year OS was 82.5%, 68.3%, 38.7%, and 25.8% for MLNR 0, 1, 2, and 3 ($P = 0.001$, $P < 0.001$ and $P = 0.038$, respectively) (Figure 2).

The performance of the 6th and 7th edition UICC N staging systems and the MLNR staging, as assessed by the linear trend χ^2, likelihood ratio χ^2, and the AIC test, is described in Table 3. Compared with the 6th and 7th edition UICC N staging systems, the MLNR staging had better homogeneity (higher likelihood ratio χ^2 score), discriminatory ability, and monotonicity of gradients (higher linear trend χ^2 score). Furthermore, the MLNR staging had a smaller AIC value, representing the optimum prognostic stratification.

Table 3 Comparison of the performance of the 6th and 7th edition International Union Against Cancer node staging systems and the metastatic lymph node ratio staging system

Classification	Subgroups	Linear trend χ^2	Likelihood ratio χ^2	AIC
6th ed UICC N staging	N 0, 1, 2, 3	117.751	141.517	7364.073
7th ed UICC N staging	N 0, 1, 2, 3	138.342	146.796	7325.731
MLNR staging	MLNR 0, 1, 2, 3	203.476	219.912	7240.017

UICC: International Union Against Cancer; N: Node; MLNR: Metastatic lymph node ratio; AIC: Akaike information criterion.

Finally, we used the ROC curves of the three aforementioned N staging systems to calculate the AUC and thus to assess the accuracy of each system's predictive ability for gastric cancer patients who underwent radical resection (Figure 3). The AUC was 0.692 for the 6th edition UICC N staging, 0.705 for the 7th edition UICC N staging, and 0.754 for the MLNR staging, indicating that the MLNR staging was superior to the 6th and 7th edition UICC N staging systems and could be used as a more precise prognostic staging tool for gastric cancer patients.

DISCUSSION

Because the 6th edition UICC TNM staging system is simple, reliable, and reproducible, it is currently used all over the world. For cases in which < 15 lymph nodes are examined, N stage may be incorrect because of stage migration. A method for bypassing this problem is to consider the ratio between metastatic and examined lymph nodes. Studies have demonstrated that staging by the MLNR is superior to staging by the absolute number of metastatic lymph nodes (such as in the 6th edition UICC N staging) for predicting prognosis of gastric cancer patients[14,26,27]. Furthermore, some reports have demonstrated that the 7th edition UICC N staging is more suitable for prognosis than the 6th edition system[18,28]. For example, although all the patients in our study underwent D2 gastrectomy with R0 resection, the number of lymph nodes recovered in the majority of patients (63.1%) was no more than 15, and therefore, in these patients, the N stage cannot be classified as N3 according to the 6th edition UICC staging system. On the other hand, in the 7th edition system, patients may be classified as N3 as long as the number of retrieved lymph nodes is more than 7, and thus, this revised edition system may reduce stage migration. Whether the 7th edition UICC N staging is optimal is still unknown. To our knowledge, although a few documents claim that the 7th edition UICC N staging is superior to the 6th edition system, there are no formal studies

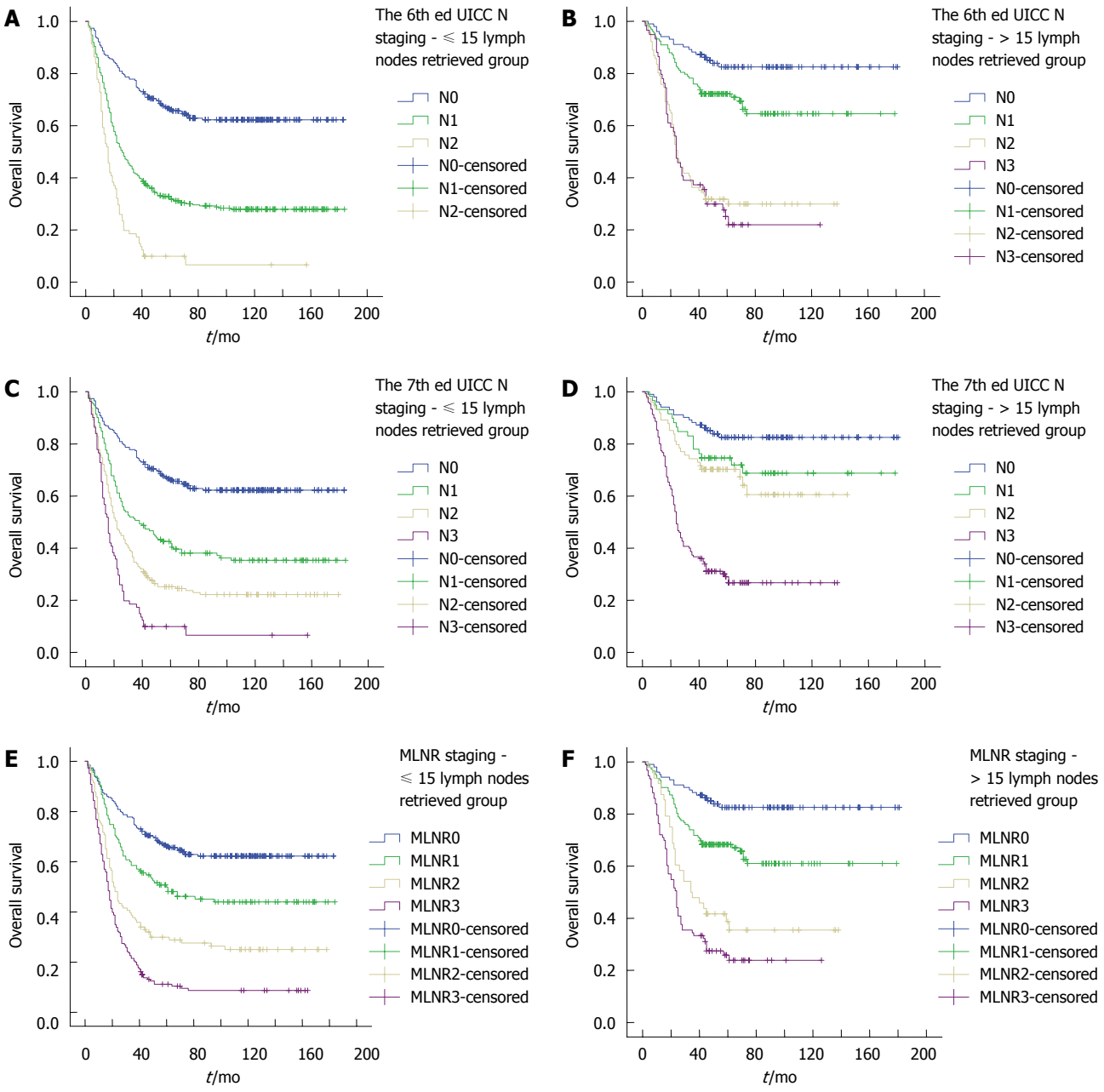

Figure 2 Analysis of the 6th (A and B) and 7th (C and D) International Union Against Cancer N staging systems and metastatic lymph node ratio staging (E and F) stratified according to the number of lymph nodes retrieved. A: The 6th International Union Against Cancer (UICC) N staging in the ≤ 15 lymph nodes retrieved group: N0 *vs* N1, $P < 0.001$; N1 *vs* N2, $P < 0.001$; B: The 6th UICC N staging in the > 15 lymph nodes retrieved group: N0 *vs* N1, $P = 0.008$; N1 *vs* N2, $P < 0.001$; N2 *vs* N3, $P = 0.720$; C: The 7th UICC N staging in the ≤ 15 lymph nodes retrieved group: N0 *vs* N1, $P < 0.001$; N1 *vs* N2, $P = 0.001$; N2 *vs* N3, $P < 0.001$; D: The 7th UICC N staging in the > 15 lymph nodes retrieved group: N0 *vs* N1, $P = 0.070$; N1 *vs* N2, $P = 0.433$; N2 *vs* N3, $P < 0.001$; E: Metastatic lymph node ratio (MLNR) stage in the ≤ 15 lymph nodes retrieved group: MLNR0 *vs* MLNR1, $P = 0.001$; MLNR1 *vs* MLNR2, $P < 0.001$; MLNR2 *vs* MLNR3, $P < 0.001$; F: MLNR stage in the > 15 lymph nodes retrieved group: MLNR0 *vs* MLNR1, $P = 0.001$; MLNR1 *vs* MLNR2, $P < 0.001$; MLNR2 *vs* MLNR3, $P = 0.038$.

that have examined the superiority of the MLNR to the 7th edition UICC N staging to date.

In this study, the MLNR was one of the most important prognostic factors of gastric cancer mortality. The MLNR provided a better classification of patient prognostic risk profiles than the 6th and 7th edition UICC N classification systems, particularly in the analysis stratified by the number of lymph nodes retrieved. The MLNR shows a clear advantage over the 6th and 7th edition UICC N staging systems for the following reasons: first, in univariate analysis, the log-rank χ^2 associated with the MLNR ($\chi^2 = 281.341$) was larger than that of the 6th and 7th edition UICC N staging systems ($\chi^2 = 160.982$ and $\chi^2 = 168.281$, respectively), indicating a higher statistical significance (Table 1); second, in multivariate analysis, the HR was higher in the MLNR (HR = 1.330, 95% CI: 1.149-1.538) staging than in the 7th edition UICC N staging (HR = 1.218, 95% CI: 1.037-1.430) (Table 2); third, although the 6th and 7th edition UICC N classifications discriminated well between each group,

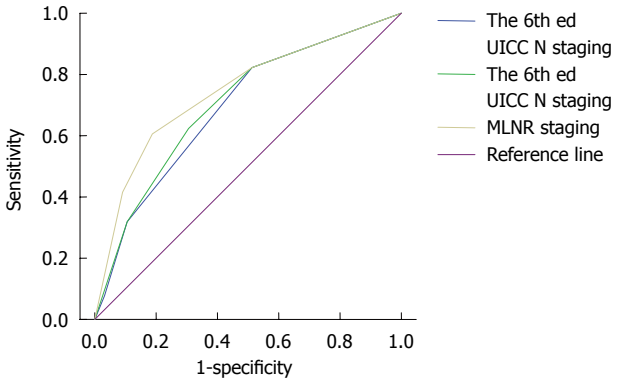

Figure 3 Receiver operating characteristics curve of the 6th and 7th edition International Union Against Cancer N staging systems and metastatic lymph node ratio staging for predicting survival of gastric cancer patients with radical resection. Area under the curve (AUC) of 1.0 represents a "perfect" diagnostic test that lacks false negative and false positive results. The AUC for the 6th and 7th edition International Union Against Cancer (UICC) N staging systems and metastatic lymph node ratio (MLNR) staging was 0.692, 0.705 and 0.754, respectively.

the MLNR provided a better classification of patient prognostic risk profiles than the pN stage, particularly in the analysis stratified by the number of lymph nodes retrieved (Figure 2); fourth, the MLNR staging had better homogeneity (higher likelihood ratio χ^2 score, 219.912 *vs* 146.796 *vs* 141.517), discriminatory ability, and monotonicity of the gradients (higher linear trend χ^2 score, 203.476 *vs* 138.342 *vs* 117.751) and a smaller AIC value (7240.017 *vs* 7325.731 *vs* 7364.073) (Table 3); and finally, the AUC under the ROC curve was larger in the MLNR staging (0.754) than in the 6th and 7th edition UICC N staging systems (0.692 and 0.705, respectively), indicating that MLNR staging was superior to the 6th and 7th edition UICC N staging methods and could be used as a more precise prognostic staging tool for gastric cancer patients.

According to Ueno *et al*[29], the performance of a staging system can be evaluated as follows: homogeneity within subgroups (small differences in survival among patients with the same stage), discriminatory ability between different groups (large differences in survival among patients in different stages), and monotonicity of the gradients shown in the correlation between stages and survival rates (within the same system, patients in earlier stages have longer survival than those in later stages). In our study, the MLNR staging had better homogeneity (higher likelihood ratio χ^2 score), discriminatory ability, and monotonicity of the gradients (higher linear trend χ^2 score) than did the 6th and 7th edition UICC N staging systems. Furthermore, in our study, the MLNR staging had a smaller AIC value, indicating that the MLNR staging has the optimum prognostic stratification and smallest loss of information for predicting outcome[30,31]. Additionally, the AUC under the ROC curve was larger in the MLNR staging than the aforementioned two N staging systems. These results demonstrate that the MLNR staging has better prognostic stratification and more precise prediction than do the 6th and 7th edition TNM staging systems.

Although the body of literature regarding the MLNR is growing, many studies have been performed using diverse patient groups and different surgical techniques. The cut-points for the MLNR have not necessarily been discussed adequately or validated in alternative data sets. We believe that systematic MLNR analyses of multi-institutional, randomized patient data with validation in similar independent data sets are required to clearly demonstrate the importance of the MLNR. Although the current UICC TNM staging system is the most basic and prevalent for predicting the survival of gastric cancer patients with radical resection, we believe that it will be essential to consider a staging system that includes accurate prognostic variables such as the MLNR in the near future. For all these reasons, the potential advantages of incorporating the MLNR in staging systems should be investigated in large, prospective data sets.

In conclusion, our study compared three lymph node based N staging systems for gastric cancer patients who underwent radical resection and D2 lymphadenectomy and then demonstrated that the MLNR categories could define gastric cancer prognosis more adequately and precisely than the 6th and 7th edition UICC N categories. We propose that nodal ratios should be considered as an alternative to the current UICC N staging.

COMMENTS

Background

In recent years, more new cases of gastric cancer are diagnosed in China each year than in any other country. The most powerful and reliable factors which have been widely used are the tumor-node-metastasis classification proposed by the International Union Against Cancer (UICC).

Research frontiers

Although UICC N staging is a convenient and reproducible method for precise staging, it demands the examination of at least 15 lymph nodes. If the number of dissected and examined lymph nodes is small or large, downmigration or upmigration of N stage may occur. The metastatic lymph node ratio (MLNR), defined as the number of positive lymph nodes divided by the number of lymph nodes retrieved, has been considered as an alternative to the absolute number of positive lymph nodes.

Innovations and breakthroughs

In the year 2010, staging of lymph node metastasis in gastric cancer has changed in both the UICC 7th edition staging system and in the Japanese Gastric Cancer Association 14th edition system to depend solely on the number of metastatic nodes found. Some authors have demonstrated that the 7th edition UICC staging system was superior to the 6th edition in aspects of homogeneity and discriminatory ability with prognostic value. However, there has been no formal proposal to date focused on comparing the prognostic significance between the MLNR and the 7th edition UICC N staging systems. In the present article, we investigated whether patients with gastric cancer can be classified into meaningful risk categories based on LNR, by comparing this staging with the 7th edition UICC N staging.

Applications

This study compared three lymph node based N staging systems for gastric cancer patients with radical resection and D2 lymphadenectomy, and then demonstrated that the MLNR categories could define gastric cancer prognosis more adequately and precisely than the 6th and 7th edition UICC N categories. The authors suggest that nodal ratios should be considered as a countermeasure to the current UICC N staging.

Peer review

This is a large study of 1042 gastric cancer patients undergoing radical resection plus D2 lymphadenectomy, with a mean follow-up of 56 mo. The authors have analyzed patient outcomes in considerable depth, their data is well characterized. They provide an in depth analysis of factors contributing to survival and have utilized multivariate analysis in doing this. The information in the manuscript is highly relevant and useful.

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Portal vein cannulation: An uncommon complication of endoscopic retrograde cholangiopancreatography

Evangelos Kalaitzakis, Nicholas Stern, Richard Sturgess

Evangelos Kalaitzakis, Nicholas Stern, Richard Sturgess, Digestive Disease Unit, Department of Gastroenterology, Aintree University Hospitals, Liverpool, L9 7AL, United Kingdom
Author contributions: Kalaitzakis E drafted the paper; all authors contributed to conception and analysis of clinical data, critical revision of the draft, and approval of the final version of the paper; and Sturgess R contributed to supervision.
Correspondence to: Evangelos Kalaitzakis, MD, PhD, Digestive Disease Unit, Department of Gastroenterology, Aintree University Hospitals, Liverpool, L9 7AL, United Kingdom. evangelos.kalaitzakis@medicine.gu.se
Telephone: +44-151-5298157 Fax: +44-151-5292490
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Abstract

Portal vein cannulation is a rare complication of endoscopic retrograde cholangiopancreatography (ERCP). It has been reported that it usually occurs after endoscopic sphincterotomy, whereas in cases without prior sphincterotomy, the presence of portobiliary fistulas has been shown. Here, we present a case in which cannulation of the portal vein occurred despite careful wire-guided cannulation and the absence of sphincterotomy. Although fatal cases of cerebral and pulmonary air and/or bile embolism have been reported in patients with combined portal and hepatic vein trauma after ERCP and sphincterotomy, isolated portal vein cannulation, as in the current case, does not usually result in mortality or serious morbidity. However, awareness of this rare complication is important so that no further intervention is performed.

Key words: Endoscopic retrograde cholangiopancreatography; Complications; Portal vein cannulation

Peer reviewer: Ibrahim A Al Mofleh, Professor, Department of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

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TO THE EDITOR

A 68-year-old man presented acutely with a 2-wk history of right upper quadrant (RUQ) pain, fever, and dark urine. Physical examination revealed mild RUQ tenderness. Investigations demonstrated deranged liver function tests with raised alkaline phosphatase (481 U/L, reference < 130 U/L), alanine aminotransferase (371 U/L, reference < 55 U/L) and bilirubin (55 μg/L, reference < 22 μg/L). C-reactive protein was elevated (195 mg/L, reference < 10 mg/L) but full blood count and renal profile were normal. Transabdominal ultrasound scan showed dilated intrahepatic bile ducts and multiple calculi in the gallbladder. Endoscopic retrograde cholangiopancreatography (ERCP) was undertaken. The papilla was edematous (prior to instrumentation), which made cannulation difficult. When wire-guided cannulation was achieved, injection of 10 mL contrast failed to opacify the biliary tree (Figure 1). The procedure was aborted and abdominal computed tomography showed air in the portal venous system (Figure 2). The patient remained asymptomatic and was hemodynamically stable. He was observed as an inpatient, and 4 d later had a repeat ERCP. The common bile duct was cannulated and a cholangiogram showed multiple filling defects consistent with stones, but no opacification of the portal venous system occurred. All stones were removed after endoscopic sphincterotomy and the patient was referred for elective laparoscopic cholecystectomy.

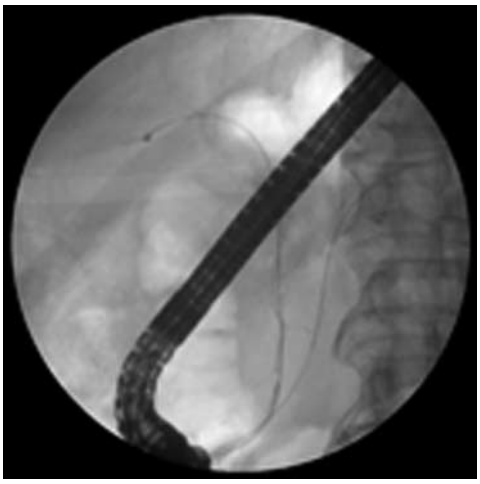

Figure 1 Sphincterotome in the portal vein after guide-wire cannulation. A two-wire technique was used as, initially, the pancreatic duct was cannulated.

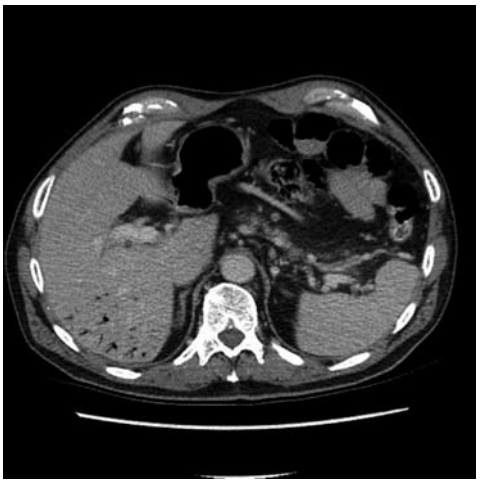

Figure 2 Abdominal computed tomography scan obtained following index endoscopic retrograde cholangiopancreatography showing air in the portal venous system.

Opacification or deep cannulation of the portal vein is a rare complication of ERCP, which occurs in 1 of 6000-8000 procedures[1]. It has been reported to occur mainly, but not solely, in patients with pancreatic cancer[1-5]; usually after pre-cut and/or conventional sphincterotomy[1,3]. Portal vein cannulation may occur as a result of direct trauma to the papilla or mucosal and vascular laceration[1]. In cases in which sphincterotomy was not performed, investigators have noted the presence of portobiliary fistulas due to presumed tumor infiltration[2] or erosion by abscesses[4]. In the current case, cannulation of the portal vein occurred despite careful wire-guided cannulation and absence of sphincterotomy. Although fatal cases of cerebral and pulmonary air and/or bile embolism have been reported in patients with combined portal and hepatic vein trauma after ERCP and sphincterotomy[6], isolated portal vein cannulation, as in the current case, has not been reported to result in mortality or serious morbidity[5]. However, in the event of large defects in the portal vein with serious bleeding, balloon tamponade or stenting with a fully covered stent can be performed, prior to considering surgical repair. Finally, awareness of this rare complication is important, so that it is readily recognized by endoscopists, and no further intervention (e.g., sphincterotomy or stent insertion into the portal vein) is performed.

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Raquel Almeida, Dr., MS, Institute of Molecular Pathology and Immunology, University of Porto, Rua Dr Roberto Frias s/n, Porto 4200, Portugal

Akira Andoh, MD, Department of Internal Medicine, Shiga University of Medical Science, Seta Tukinowa, Otsu 520-2192, Japan

Luis Bujanda, PhD, Professor, Department of Gastroenterology, CIBEREHD, University of Country Basque, Donostia Hospital, Paseo Dr. Beguiristain s/n, 20014 San Sebastián, Spain

Rosemary Joyce Burnett, MPH, Department of Epidemiology National School of Public Health, University of Limpopo, Medunsa Campus PO Box 173, MEDUNSA, Pretoria 0204, South Africa

Clifford S Cho, MD, Assistant Professor, Department of Surgery, Section of Surgical Oncology, University of Wisconsin School of Medicine and Public Health, H4/724 Clinical Sciences Center, 600 Highland Avenue, Madison, WI 53792-7375, United States

Hugh J Freeman, Professor, Department of Medicine, University of British Columbia, UBC Hospital2211 Wesbrook Mall, Vancouver, BC V6T 1W5, Canada

Morten Frisch, Dr., MD, PhD, DSc, Department of Epidemiology Research, Statens Serum Institut, Building 206, Room 317, 5 Artillerivej, DK-2300 Copenhagen S, Denmark

Diego Garcia-Compean, MD, Professor, Faculty of Medicine, University Hospital, Department of Gastroenterology, Autonomous University of Nuevo Leon, Ave Madero y Gonzalitos, 64700 Monterrey, NL, México

Evangelos Kalaitzakis, MD, PhD, Associate professor, Institute of Internal Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg 41345, Sweden

Leonidas G Koniaris, Professor, Alan Livingstone Chair in Surgical Oncology, 3550 Sylvester Comprehensive Cancer Center (310T), 1475 NW 12th Ave., Miami, FL 33136, United States

Ezio Laconi, MD, PhD, Professor of General Pathology, Department of Sciences and Biomedical Technologies, Unit of Experimental Pathology, University of Cagliari, Via Porcell, 4-IV Piano, 09125 Cagliari, Italy

Emanuel K Manesis, MD, Professor of Medicine, Athens University School of Medicine, Liver Unit, Euroclinic, 19 Mavromateon Street, Athens 1034, Greece

Kenji Miki, MD, Department of Surgery, Showa General Hospital, 2-450 Tenjin-cho, Kodaira, Tokyo 187-8510, Japan

Mark S Pearce, Dr., Paediatric and Lifecourse Epidemiology Research Group School of Clinical Medical Sciences, University of Newcastle, Sir James Spence Institute, Royal Victoria Infirmary, Newcastle upon Tyne, NE1 4LP, United Kingdom

Guida Portela-Gomes, MD, PhD, Professor, Faculty of Medicine, University of Lisbon, Rua Domingos Sequeira-128, Estoril 2765-525, Portugal

Lima Ribeiro Marcelo, PhD, Clinical Pharmacology and Gastroenterolgy Unit, Laboratory of Microbiology and Molecular Biology, Sao Francisco University Medical School, Av Sao Francisco de Assis, 218, Braganca Paulista-SP 12916-900, Brazil

Casadesus Rodriguez Damian, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City 999075, Cuba

Ekihiro Seki, MD, PhD, Department of Medicine, University of California San Diego, Leichag Biomedical Research Building Rm 349H, 9500 Gilman Drive MC No. 0702, La Jolla, CA 92093-0702, United States

Arne Søreide Jon, Professor, MD, PhD, FACS, Department of Surgery, Stavanger University Hospital, N-4068 Stavanger, Norway

Thomas Wild, Dr., MD, Department of Surgery, Paracelsus Medical University, Feldgasse 88, Kapellerfeld 2201, Austria

Mitsunori Yamakawa, Professor, Department of Pathological Diagnostics, Yamagata University, Faculty of Medicine, 2-2-2 Iida-Nishi, Yamagata 990-9585, Japan

Yuichi Yoshida, MD, PhD, Assistant Professor, Department of Gastroenterology and Hepatology, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Katsutoshi Yoshizato, PhD, Academic Advisor, Phoenix Bio Co., Ltd., 3-4-1 Kagamiyama, Higashihiroshima 739-0046, Japan

Run Yu, MD, PhD, Division of Endocrinology, Diabetes and Metabolism, Cedars-Sinai Medical Center, 8700 Beverly Blvd, B-131, Los Angeles, CA 90048, United States

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AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

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No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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