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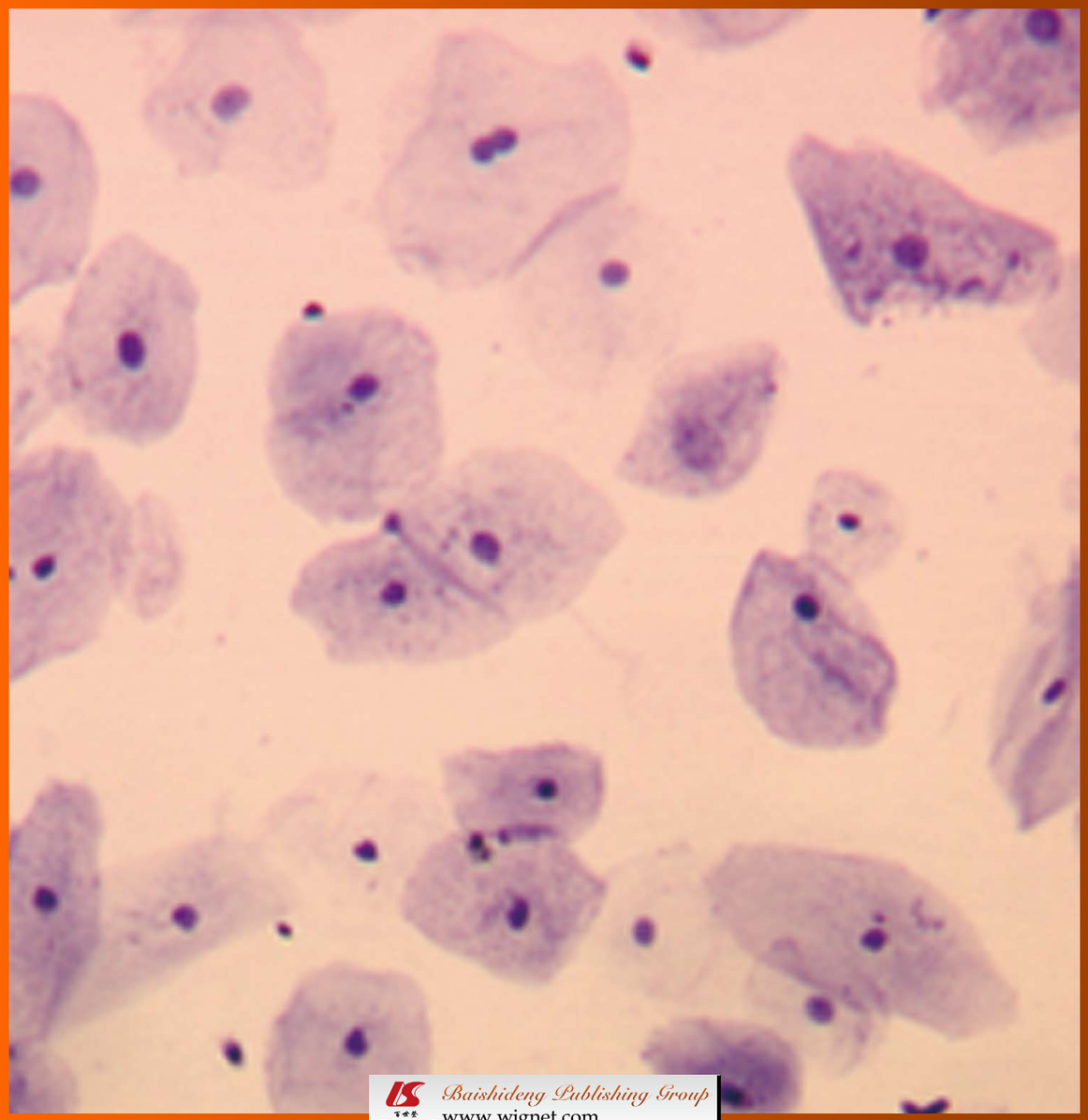

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EDITING
Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-5908-0039
Fax: +86-10-8538-1893
E-mail: wjg@wjgnet.com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

Chronic proctalgia and chronic pelvic pain syndromes: New etiologic insights and treatment options

Giuseppe Chiarioni, Corrado Asteria, William E Whitehead

Giuseppe Chiarioni, Division of Gastroenterology of the University of Verona, Azienda Ospedaliera Universitaria Integrata di Verona, Valeggio sul Mincio Hospital, 37067 Valeggio sul Mincio, Verona, Italy
Corrado Asteria, Department of Surgery and Orthopedics, General Surgery Unit, Azienda Ospedaliera Carlo Poma di Mantova, Asola General Hospital, 46041 Asola, Mantova, Italy
William E Whitehead, Division of Gastroenterology and Hepatology, Division of Urogynecology, and Center for Functional Gastrointestinal and Motility Disorders, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7080, United States
Author contributions: Chiarioni G and Whitehead WE contributed equally to the conceiving of the designing and the drafting of the manuscript; Asteria C provided criticism and suggestion of high intellectual content.
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Correspondence to: Giuseppe Chiarioni, MD, Division of Gastroenterology of the University of Verona, Azienda Ospedaliera Universitaria Integrata di Verona, Valeggio sul Mincio Hospital, 37067 Valeggio sul Mincio, Verona, Italy. chiarioni@tin.it
Telephone: +39-045-6338548 Fax: +39-045-7950188
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Abstract

This systematic review addresses the pathophysiology, diagnostic evaluation, and treatment of several chronic pain syndromes affecting the pelvic organs: chronic proctalgia, coccygodynia, pudendal neuralgia, and chronic pelvic pain. Chronic or recurrent pain in the anal canal, rectum, or other pelvic organs occurs in 7% to 24% of the population and is associated with impaired quality of life and high health care costs. However, these pain syndromes are poorly understood, with little research evidence available to guide their diagnosis and treatment. This situation appears to be changing: A recently published large randomized, controlled trial by our group comparing biofeedback, electrogalvanic stimulation, and massage for the treatment of chronic proctalgia has shown success rates of 85% for biofeedback when patients are selected based on physical examination evidence of tenderness in response to traction on the levator ani muscle-a physical sign suggestive of striated muscle tension. Excessive tension (spasm) in the striated muscles of the pelvic floor appears to be common to most of the pelvic pain syndromes. This suggests the possibility that similar approaches to diagnostic assessment and treatment may improve outcomes in other pelvic pain disorders.

Key words: Biofeedback; Chronic pelvic pain; Chronic proctalgia; Coccygodynia; Levator ani syndrome; Pudendal neuralgia

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INTRODUCTION

Chronic or frequently recurring pain in the anal canal, rectum, or pelvis is a prevalent symptom that affects an estimated 6.6% of the population[1]. Although only 1/3 of people with such pains consult physicians, they nev-

ertheless report significant impairment in quality of life, work absenteeism, and psychological distress. However, despite its prevalence and impact, relatively little research has been published which addresses its epidemiology, pathophysiology, and treatment; and pelvic and rectal pain is widely considered frustrating to diagnose and treat. This may soon change: Our group recently reported a randomized controlled trial[2] in which we compared different approaches to treating chronic proctalgia and showed that the results were excellent 85% success rate-if the patients were appropriately screened. This study also provided new insights into the pathophysiology of chronic proctalgia and stimulated renewed interest in anorectal pain syndromes. The aims of this review are to critically assess what is known about the diagnosis and treatment of the most common forms of anorectal and pelvic pain, namely chronic proctalgia, chronic pelvic pain, coccygodynia, and pudendal neuralgia. This review is meant to help Gastroenterologists and Colorectal Surgeons when dealing with complex consultation on pelvic pain syndromes. It is mostly focused on chronic proctalgia and issues of differential diagnosis with other pelvic pain syndromes.

One of the challenges in caring for patients with anorectal and pelvic pain is that a number of inflammatory and structural etiologies must be considered. The organic diseases that are most commonly involved in chronic anorectal and pelvic pain are cryptitis, fissure, abscess, hemorrhoids, solitary rectal ulcer, inflammatory bowel disease, and rectal ischemia[3]. One should also consider chronic prostatitis and pelvic endometriosis as potential contributors to chronic pelvic pain[3]. Although the differential diagnosis is large and unfortunately poorly standardized, our experience[2] suggests that no organic disease explanation will be found in approximately 85% of patients presenting to gastroenterologists with chronic anorectal or pelvic pain. We screened 227 patients referred for unremitting, chronic rectal pain with a diagnostic evaluation that included digital rectal examination, colonoscopy, pelvic ultrasound and surgical consultation in all patients, plus gynecology and urology referrals in selected cases[2]. This extensive work-up identified only 33 patients (15%) with a probable organic disease accounting for their symptoms. Thus, for most patients with chronic anorectal or pelvic pain, the origin of the pain is uncertain and the relevant pathophysiological mechanisms are unclear. These are commonly defined as "functional" chronic anorectal and pelvic pain syndromes since no structural and anatomical disease was found. These functional pain syndromes constitute the main subject of this review.

CHRONIC PROCTALGIA

Chronic proctalgia is a general term for chronic or recurring pain in the anal canal or rectum[3]. Other names considered synonymous with chronic proctalgia are levator ani syndrome, puborectalis syndrome, chronic idiopathic perineal pain, pyriformis syndrome, and pelvic tension myalgia. Thiele, one of the first researchers to investigate this pain syndrome, called it coccygodynia, although he acknowledged that the pain was not in the coccyx[4]. To provide greater consistency in the diagnosis and labeling of anorectal pain syndromes, the Rome Ⅲ criteria[3] define chronic proctalgia as chronic or recurrent rectal pain or aching lasting at least 20 min, in the absence of structural or systemic disease explanations for these symptoms[3]. Pain duration of at least 20 min is a key feature since shorter episodes of pain are suggestive of proctalgia fugax, which is defined as a sudden, severe pain in the anorectal region lasting less than 20 min and then disappearing completely[3]. Proctalgia fugax may recur, but episodes are rare. Proctalgia fugax is believed to have a different etiology to chronic proctalgia, although there is no consensus on what causes it. Its consideration is beyond the scope of this review, which is intended to deal with chronic unremitting diseases.

Chronic proctalgia is further divided by the Rome Ⅲ criteria into two subtypes-levator ani syndrome (LAS) and unspecified functional anorectal pain-based on the presence or absence of a sensation of tenderness when the levator muscle is palpated during digital rectal examination. This classification updates the previous Rome Ⅱ classification in which LAS was designated as "highly likely" if traction on the pelvic floor produced a report of tenderness and only "possible LAS" if no tenderness was elicited[5]. Subgrouping patients with chronic proctalgia is consistent with clinical experience of different response to treatment, but distinct epidemiology and pathophysiology data are lacking[5]. Therefore, data provided mostly refer to chronic proctalgia patients as a whole.

Pathophysiology

Chronic tension or spasm of the striated muscles of the pelvic floor is commonly assumed to be the pathophysiological basis for chronic proctalgia[3,5-7], although there is no definitive evidence for this hypothesis. Inflammation of the levator or arcus tendon of the levator ani muscle has also been suggested as a cause of chronic proctalgia, since tenderness on palpation is most commonly found on the left side where the muscle inserts into the pubic ramus of the pelvis. However, contrary to this tendinitis hypothesis, local steroid injection has not been shown to be an effective treatment for chronic proctalgia[8]. In retrospective studies, many patients reported prior pelvic surgery, anal surgery and even spinal surgery as significant in the development of their pain syndrome[6,9]. Childbirth can be another precipitating factor[9]. In addition, high rates of anxiety disorders, depression, and stress are frequently reported in chronic proctalgia, and may act as significant precipitating factors in some patients[6,10].

Except for the exclusion of organic diseases, tests of anorectal physiology and imaging studies were traditionally considered to be of little diagnostic or prognostic

value[11,12]. Increased anal canal resting pressures tested by anorectal manometry were sometimes reported, but results were inconsistent. Grimaud and coworkers reported that LAS was associated with anal sphincter hypertonia and disordered defecation on dynamic proctography in a study of 12 patients, but this was not confirmed in a larger prospective study of 60 patients by Ger and coworkers[11,13] . Ger *et al*[11] reported that LAS was associated with paradoxical contraction of the pelvic floor muscles on straining as evidenced by anal electromyography or defecography. However, all these studies were potentially biased by small size, mixed patient population, and poor patient selection[3,5]. In addition, a number of structural disorders (descending perineum, rectocele, mucosal prolapse and pelvic floor dyssyenrgia) have been reported in small studies[6,11-13].

In a recent study, Hompes *et al*[14] reported on 59 patients referred to a Pelvic Floor Clinic for chronic functional anorectal pain who were tested by means of defecating proctography, anorectal manometry, anal ultrasound, and in selected cases, rectal examination under anesthesia. The same diagnostic protocol was applied to 543 rectal prolapse patients complaining of obstructed defecation and to a control group of patients with fecal incontinence. In the control group with fecal incontinence, pain was reported in 50% of patients but was a non-dominant symptom. Anorectal manometry failed to show any difference among groups. Rectal morphology examinations demonstrated high grade internal rectal prolapse in 59% of pain patients, which was often associated with symptoms of obstructed defecation. The authors concluded that rectal prolapse commonly underlies chronic proctalgia, particularly when obstructed defecation is present. However, the severity of prolapse did not correlate with pain intensity, leaving pain pathophysiology unclear[14]. In addition, chronic idiopathic rectal pain is sometimes reported as a complication of corrective surgery for rectal prolapse[14].

An innovative pathophysiology explanation for chronic proctalgia was recently reported by our group in a large, prospective, randomized controlled trial comparing biofeedback, electrogalvanic stimulation (EGS), and digital massage of the levator muscles for the treatment of chronic proctalgia. In this study, 157 patients with chronic proctalgia (confirmed by Rome Ⅱ criteria) were studied by anorectal manometry and a balloon evacuation test at baseline and again after 3 mo of treatment[2]. Based on a priori exclusion criteria, patients reporting symptoms consistent with either irritable bowel syndrome or functional constipation were not enrolled in the study. In patients reporting tenderness on palpation of the levator muscles (Rome Ⅱ: highly likely LAS, Rome Ⅲ: LAS), physiologic features of dyssynergic defecation (i.e., paradoxical contraction or failure to relax the pelvic floor on straining) were seen in approximately 85% of subjects in the absence of symptoms of constipation. Conversely, in patients who denied tenderness when the levators were palpated during digital rectal examination, inability to relax pelvic floor muscles when straining was an uncommon finding (19%). Dyssynergic defecation was a strong predictor of successful treatment outcome. These observations led us to conclude that the physiologic mechanisms responsible for LAS and dyssynergic defecation are similar[2].

This study also showed that the inability to relax pelvic floor muscles when straining to defecate may occur without symptoms of constipation, even though it is commonly assumed that dyssynergic defecation invariably results in obstructed defecation. Factors that interact with pelvic floor physiology to determine which symptoms develop-either pain or constipation-are left unanswered by our study and deserve further investigations. Also, we were not able to provide a physiological explanation for unspecified functional anorectal pain (i.e., anorectal pain without tenderness on digital palpation), which may represent a heterogeneous group of patients. Our study suggests, however, that adding a simple balloon evacuation test with a disposable Foley catheter to the diagnostic work up of chronic proctalgia patients enables one to select subjects that are more likely to benefit from pelvic floor rehabilitation.

Clinical presentation

Chronic proctalgia is often described by patients as a dull ache or pressure sensation in the rectum that is exacerbated by prolonged sitting and relieved by standing or lying down[3,5]. This pain rarely occurs at night; rather, it usually begins in the morning and increases in severity throughout the day. The pain may be precipitated by long-distance car travelling, stress, sexual intercourse and defecation[6,7]. During digital rectal examination, the examining finger is moved from the coccyx posteriorly to the symphysis pubis anteriorly[5,7]. For unexplained reasons, tenderness is often non-symmetric, being greater on the left side than on the right[5]. When performing digital rectal examination, the examiner should pause after inserting their finger into the rectum before applying traction on the levator muscles to avoid false positive results. In our experience, repeating the posterior traction on the levator muscle on the same exam is also useful to check for reproducibility and to avoid false positive results.

Diagnostic assessment

Although our recent study provides new insights into the pathophysiology of chronic proctalgia, these observations require validation by other laboratories before they can be incorporated as diagnostic criteria. The diagnosis of chronic proctalgia still relies on (1) clinical symptoms of recurring or chronic pain or aching in the anal canal or rectum with episodes lasting 20 min or longer[3], and (2) exclusion of alternative disease explanations for these symptoms by multiple diagnostic tests and consultations by other specialists. In addition, digital rectal examination should be performed to ascertain whether the patient reports tenderness when traction is applied to the levator ani muscles because this diagnostic sign

is a strong predictor of whether the patient is likely to benefit from treatments directed at relaxing pelvic floor muscles.

Treatment

No single treatment has been reported to be consistently effective in chronic proctalgia[3,7], and management can be a frustrating endeavor for both patients and physicians[11]. The first-line treatment most commonly provided is reassurance that the pain is of benign origin and is not suggestive of malignancy[7,9]. No data are available on the impact of reassurance, but education and counseling are often incorporated as a component of treatment.

Digital massage of the puborectalis sling, intended to relax tense muscles, was one of the first treatments proposed for chronic proctalgia[9]. Massage of the puborectalis muscle should be performed in a firm manner with the affected side massaged up to 50 times, depending on the patient's tolerance. Some claim that if the massage is not uncomfortable to the patient while being performed, it may not be effective[9]. Massage of the levator ani muscle is rarely performed as the sole therapy, with the most common adjunctive treatments being hot sitz baths or a short-term course of oral Diazepam, both of which are assumed to have myorelaxant properties. Earlier open-label studies suggested that digital massage combined with hot sitz baths and/or Diazepam were effective for relieving pain in 68% of 316 chronic proctalgia patients[15]. However, benefits seemed to fade away during long-term follow-up, and the addictive potential of Diazepam discourages long-term treatment[7].

Electrogalvanic stimulation, traditionally used by physiatrists to treat muscle spasticity[9], has also been advocated for the treatment of LAS when conservative therapy is ineffective. A low frequency oscillating current applied to the pelvic floor muscles through an anal probe, induces fasciculation and prolonged fatigue, which breaks the spastic cycle and may produce sustained symptom relief. Low frequency current has no thermal effect. No side effects have ever been reported other than mild worsening of pain on the first days of treatment. Sohn and coworkers were the first to test EGS in an open study of 80 chronic proctalgia patients[16]. They recommended a pulse frequency of 80 cycles per second with the voltage being gradually increased from zero to the point of discomfort (250-300 Volts according to patient's tolerance). Recommended treatment duration is one hour per day for 3 sessions in a ten-day period. In the Sohn study[16], 91% of patients reported good to excellent pain relief from EGS in the short-term, but no long-term follow-up was reported. This high percentage of success was never replicated by subsequent open label studies, although approximately two-thirds of patients did report short-term pain relief. Treatment protocols varied widely in terms of number and duration of sessions. Authors claimed that non-responders showed features of psychology disturbances, but no evidence was provided on the issue. However, three additional studies that investigated the long-term benefits of EGS treatment in chronic proctalgia found that only 25%-38% of patients reported persistent pain improvement[17-19].

Biofeedback treatment of LAS was first described in 1991 by Grimaud and coworkers[13]. They treated 12 patients with biofeedback techniques focused on voluntary relaxation of external anal sphincter tone. Pain disappeared in all patients after a mean of eight sessions. Subsequent studies using biofeedback were not able to replicate these results, with success rates varying from 35% to 87.5%[6,11,19]. All studies were small, none was controlled, and treatment modalities varied.

Botulinum Toxin A (BoTox A) was tested in a randomized controlled trial run in 12 patients, and no differences in rectal pain were observed between patients injected with active BoTox *versus* those injected with saline[20]. The average amount of time required to defecate a rectal balloon was actually increased after BoTox injection. The tendinitis (inflammation) hypothesis for chronic proctalgia was tested by steroid caudal block and by pelvic tender point injection of a mixture of Triamcinolone Acetonide and Lidocaine with negative results[8,11]. Sacral nerve stimulation was also reported to be beneficial in an open study involving 27 chronic proctalgia patients. However, when benefits were assessed by intent to treat analysis, pain relief was reported in less than 50% of subjects[21].

A major drawback in assessing the literature on chronic proctalgia treatment is the huge variation in inclusion criteria, outcome criteria, and follow-up intervals. Additional limitations are small sample sizes and lack of an appropriate control group. The few quasi-randomized studies had control groups that included subjects who received more than one treatment and patients not responding to a former therapy[7]. To overcome these limitations, Chiarioni and coworkers recently reported a prospective, randomized controlled trial of 157 chronic proctalgia patients to investigate the comparative effectiveness of the 3 most commonly prescribed treatments: biofeedback to teach pelvic floor muscle relaxation, EGS, and digital massage of the levator muscles[2]. A physiological assessment including manometry and balloon defecation was carried-out at baseline and at 1-3 mo follow-up. In addition, self-reported stool frequency was assessed at baseline and at 6-mo follow-up. The primary outcome was subjective reporting of adequate pain relief by the patient. Secondary outcomes included subjective pain improvement on an ordinal scale, number of days per month with rectal pain, and visual analog scale ratings of pain. According to Rome II criteria, proctalgia patients were subgrouped into highly likely LAS and possible LAS based on the presence or absence of levator tenderness at digital rectal exam, and randomization to treatment groups was stratified so that each treatment group contained a similar number of patients with a highly likely diagnosis of LAS.

At one-month follow-up, biofeedback was significantly more effective than EGS and massage by intent-to-treat analysis, with adequate relief of pain reported by 59.6% *vs* 32.7% *vs* 28.3% for biofeedback, EGS, and massage, respectively. Benefits were maintained throughout follow-up (12 mo) and no side effects were reported with any treatment. When results were further investigated in subgroups of patients, no treatment was effective in possible LAS patients (Rome Ⅲ unspecified functional anorectal pain). However, among patients with highly likely LAS (Rome Ⅲ levator ani syndrome) adequate relief was reported by 87% for biofeedback, 45% for EGS and 22% for massage at 1 mo follow-up. Improvements were maintained for the whole follow-up. The superiority of biofeedback was supported by all the secondary outcome measures including number of days per month with pain, which decreased from 14.7 per month to 3.3 per month for biofeedback, 8.9 for EGS, and 13.3 for massage[2].

Physiological measurements revealed that the mechanism for achieving adequate pain relief was an improvement in pelvic floor function from being unable to relax anal canal pressures on straining to being able to do so and/or an improvement on the balloon evacuation test from being unable to pass a 50 mL balloon to being able to do so[2]. This interpretation of the mechanism of action was confirmed by a post-hoc analysis showing that 94.2% of those who improved pelvic floor dysfunction on one or both of these measures reported adequate pain relief, while only 13.6% of those who did not improve pelvic floor function reported positive therapy outcome regardless of the treatment provided. In addition, stool frequency increased from baseline to post-treatment in responders, even in the absence of a former complaint of constipation. This study led us to conclude that biofeedback is an effective treatment for LAS, and EGS is somewhat effective. However, the minority of proctalgia patients affected by unspecified functional anorectal pain are still left without a satisfactory treatment option. In this regard, depression and anxiety are both frequently reported in non-responsive proctalgia patients[6,10]. Brain processing of pain may be altered in functional gastrointestinal disorders, but data in proctalgia patients are lacking[22]. In addition, no trial has actually evaluated the effect of either psychotherapy intervention or psychotropic drugs in proctalgia patients. Finally, there is no evidence that surgery can help these severely disabled patients. Invasive interventions should be avoided in the absence of a clearer etiologic understanding of non-responsive proctalgia patients[3].

COCCYGODYNIA

Coccygodynia is defined as pain arising in or around the coccyx, usually triggered by prolonged sitting on hard surfaces[23]. The pain is considered chronic when it lasts more than two months and it is commonly reported after repetitive trauma or childbirth[23,24]. Coccygodynia may also be of idiopathic origin or secondary to lumbar disc degeneration[23-25]. It is also rarely reported as a complication of epidural injection of anesthetic or of various rectal and spine surgery[25].

Pathophysiology

It is up to five times more common in women than in men, and obesity seems to be a predisposing factor due to the associated pelvic rotation[23-25]. The female pelvic anatomy may also predispose to coccygodynia by leaving the coccyx more exposed to traumatic injury. The exact etiologic mechanism/s associated with coccygodynia are still obscure. Chronic spasm of the pelvic floor exerting a painful tension on a stiff coccyx has been traditionally considered a relevant etiologic factor, with accidental trauma acting as a trigger[23-25]. However, instability of the coccyx potentially correlated with symptom severity was then discovered in a high percentage of patients by dedicated X-Ray examination[26]. In addition, it is unclear whether pre-existing spine alterations play a role by predisposing patients to develop post-traumatic coccygodynia[26,27]. Inflammation of structures (i.e., bursitis) in close proximity to the spine has also been described as a causative factor in a minority of patients complaining of coccygodynia[27]. Depression and anxiety disorder have been reported to amplify coccygeal pain symptoms[23]. Some authors do not diagnose coccygodynia when there is an ongoing medicolegal litigation, even if it occurs following a traumatic injury[24,25].

Clinical presentation

Pain in the coccyx and in close anatomical regions (sacrum, perineum, anorectum) is the main reported symptom[23]. Epidemiologic data on coccygodynia in the general population are lacking, but coccygodynia is considered to be a rare disorder. Retrospective data suggest that coccygodynia accounts for less than 1% of all reported cases of lower back pain[24,25]. Diagnosis of coccygodynia relies heavily on history and clinical exam. Questioning the patient about previous trauma to the coccyx or childbirth trauma is a must, since according to Salvati the absence of a previous trauma makes the diagnosis unlikely[9]. In addition, patients should report worsening of pain by prolonged sitting, bending, lifting or having a restricted poor posture for long intervals[23-25]. Some patients may report that standing from a sitting position triggers the pain[23].

Diagnostic assessment

Reproducing the usual pain by pressure or manipulation of the coccyx is key to diagnosis[23]. Patients may also report mild tenderness on puborectalis posterior traction on digital rectal examination and a differential diagnosis of chronic proctalgia needs to be entertained[9,28]. However, this maneuver should never be able to provoke the usual pain. Abnormal movement of the coccyx on palpation is an additional sign to confirm the clinical suspicion of coccygodynia[25]. Dynamic X-Ray investigation may support the clinical diagnosis. The standard lateral

X-Ray investigation of the coccyx in the standing position should be supplemented with a second film taken while the patient is sitting on a hard surface possibly in a posture worsening the pain[26]. More than 50% of patients would show features of coccyx instability (either exaggerated flexion or luxation) that seem to correlate with pain severity and previous traumatic events. An additional 15% of coccygodynia patients would show features of an abnormal bone spur at the end of the tailbone (so called spicule)[26]. Spine magnetic resonance imaging (MRI) could be performed to exclude tumors or disc disease, but do not seem to add significantly to the diagnosis in coccygodynia[25].

Treatment

The initial treatment of coccygodynia is focused on avoiding potentially offending factors and includes sitting on a donut-shaped pillow or a gel cushion to reduce pressure, posture ameliorating interventions, sitz bath and on demand nonsteroidal anti-inflammatory drugs[23,24]. This treatment is commonly applied for 6-8 wk. No controlled study has investigated the therapeutic outcome of these simple measures. When initial treatment fails most authors recommend adding digital manipulation of the coccygeal ligaments as well as intrarectal manipulation of the pelvic floor muscles. Various massage and manipulation techniques have been described in open studies to decrease coccygeal pain in up to 85% of patients, particularly when combined with local steroid injection or physiotherapy[29]. A recent prospective, randomized, controlled study aimed to compare intrarectal pelvic floor muscles manipulation (3 sessions) *vs* placebo physiotherapy (sacral short wave magnetic field applied at marginal power) in 102 chronic coccygodynia patients[30]. Primary outcome was subjective decrement of more than 50% in pain intensity on a visual analog scale score at follow-up intervals of 1-6 mo. At 1 mo follow-up, 22% of patients in the manipulation group reported a significant pain decrement compared to only 12% of patients in the placebo group. Benefits persisted throughout follow-up in both groups. Manipulation was more effective in recent onset coccygodynia of post-traumatic origin not associated with instability of the coccyx. Psychosocial factors seemed to predict a poorer treatment outcome. The authors concluded that intrarectal manipulation is at least mildly effective in chronic coccygodynia and suggested either to increase the number of therapeutic sessions or to add local steroid injection to improve outcome. However, no randomized study has actually evaluated both treatment options for coccygodynia. In selected patients with severe and unresponsive coccygodynia, surgery may be considered[31].

A recent review on surgical treatment of coccygodynia reported on 24 studies, but 22 of them were retrospective case series[32]. Surgery was a treatment option in a minority of patients (approximately 19%), but mean satisfaction rate for pain relief was high (over 80% of treated patients). Some series reported a satisfactory outcome of just 54% which was attributed to patient selection bias. Mean overall complication rate was 10.9% with wound infection being the most commonly reported complication. Surgeon expertise seemed to play a role since the smallest series reported the highest procedure-related complication rates (up to 50%). The type of surgery chosen was either total or partial removal of the coccyx and this did not seem to influence outcome. However, the worst outcomes were reported in patients with a history of rectal or spinal diseases and ongoing compensation issues[32].

PUDENDAL NEURALGIA

Pathophysiology

Pudendal neuralgia is a chronic pain in the perineal area secondary to entrapment and injury to the pudendal nerve in its musculo-osteo-aponeurotic tunnel between the sacrotuberal and sacrospinal ligaments, in the absence of organic diseases that may explain this symptom[23]. Pudendal neuralgia has been rarely described as secondary to herpetic neuropathy, stretch neuropathy, and post-radiotherapy neuropathy, but pudendal nerve entrapment is by far the most common etiology[33,34]. Pudendal neuralgia is also called Alcock's canal syndrome, or pudendal canal syndrome[23].

Clinical presentation

It is commonly described as a superficial pain, burning sensation, numbness, or paresthesia in the gluteal, perineal, and/or genital areas[23]. It may be homolateral or bilateral, radiate to the pelvis and the thighs, and be associated with deep pelvic discomfort[33,34]. Pain may be worsened by sexual intercourse and initially reported as sciatic pain[33,34]. The epidemiology of pudendal neuralgia in the general population is unknown. The diagnosis is rarely considered except in highly focused Pelvic Floor Units or in specialized Urogynecologist practices. It is usually considered to be a rare entity, but it may be overdiagnosed due to the functional comorbidities associated with pudendal nerve dysfunction[33,34]. Recently, a multidisciplinary Committee reported that pudendal neuralgia may be simply diagnosed by default in the presence of pelvic, perineal, and buttock pain without evidence of organic disease at diagnostic workup[35]. Particularly controversial is its association with rectal pain, the presence of which requires differential diagnosis with chronic proctalgia[23].

Diagnostic evaluation

Clinical neurophysiology has improved our knowledge of this disorder, but a definitive diagnostic test is still not available. As in many neuropathic pain syndromes, the diagnosis of pudendal neuralgia remains primarily clinical and should be reviewed in the light of the course of the disease. In 2006, a multidisciplinary working party on pudendal neuralgia held in Nantes, France, concluded that only the operative finding of nerve entrapment

and post-operative pain relief can formally confirm the diagnosis, provided the placebo effect of surgery is excluded[35]. However, this panel of experts identified four domains of diagnostic criteria for pudendal neuralgia: (A) essential criteria, (B) complementary diagnostic criteria, (C) exclusion criteria, (D) associated signs not excluding the diagnosis. Essential criteria are particularly relevant and will be discussed in detail. (1) Pain should be limited to the innervation territory of the pudendal nerve. This excludes any pain that is limited to the coccygeal, pelvic or gluteal areas; (2) Pain is predominantly experienced while sitting, in accordance with the nerve compression etiology hypothesis. In long-standing pudendal neuralgia, pain may become continuous, but it is still worsened by the sitting position; (3) The pain rarely awakens the patient at night; (4) On clinical examination, no objective sensory impairment can be found even in the presence of paresthesia. The presence of a sensory defect should prompt investigations to exclude diseases of the sacral nerve roots and the cauda equina; and (5) Pain should be relieved by anesthetic infiltration of the pudendal nerve. This is an essential criterion, but it lacks specificity as pain related to any perineal disease may be relieved by pudendal nerve block. Moreover, a negative block does not exclude the diagnosis of pudendal neuralgia because it may have been performed inadequately (e.g., too distally). The complementary diagnostic criteria include the sensation of a rectal foreign body and the worsening of pain during defecation, both of which should prompt the physician to entertain the differential diagnosis of chronic proctalgia. Exclusion criteria for pudendal neuralgia are pain in a territory unrelated to the pudendal nerve, symptomatic pruritus instead of paresthesia, exclusively paroxysmal pain, and imaging abnormalities that could explain the symptom[35].

Treatment

Pudendal neuralgia is treated by pudendal nerve block, which is both diagnostic and therapeutic. However, data on the long-term benefits of pudendal nerve block are lacking[35]. In addition, only the operative demonstration of nerve entrapment and post-operative pain relief can formally confirm the diagnosis of pudendal neuralgia secondary to it, except for a potential placebo effect of surgery[35].

CHRONIC PELVIC PAIN IN WOMEN

Chronic pelvic pain (CPP), which is diagnosed only in women, is commonly defined as noncyclic, nonmalignant pain in any organs related to the pelvis, in the absence of pregnancy and inflammatory bowel disease, that has lasted for at least six months[36]. Pain occurring exclusively in association with menstruation (dysmenorrhea) and sexual intercourse (dyspareunia) are generally not considered to be CPP, but general agreement is lacking. Other definitions include a pain severity sufficient to cause functional disability or to require medical care[36]. Since the definition of CPP varies, it is difficult to ascertain its exact prevalence. However, the prevalence of CPP in the general population assessed by mail questionnaires among women aged 18-50 has been reported to be as high as 15% in the United States and 24% in the United Kingdom[37,38]. CPP has been estimated to account for 10% of all outpatient referrals to gynecologists and 40% of diagnostic laparoscopies, so it constitutes a significant economic burden[39,40]. No organic disease is found on laparoscopy in at least a third of women with CPP[40]. In the community, 32% of patients who consult for this symptom report high rates of anxiety and quality of life impairment as measured by the SF-36[37,38]. Consulting behavior is directly influenced by the severity of pain[39].

Pathophysiology

The etiology of CPP is considered to be complex and multifactorial[36,40]. Some gynecological diseases such as endometriosis, pelvic inflammatory disease, and interstitial cystitis may cause CPP, but gastrointestinal comorbidities are also reported in up to 1/3 of CPP patients in primary care[38,40]. A psychosomatic component of pain has also been hypothesized[36,40]. The common association of CPP with irritable bowel syndrome has led some to question whether these two diseases are actually a single clinical entity that is diagnosed differently according to the specialist consulted[41]. The etiology of CPP is poorly understood.

Diagnostic assessment

Initial evaluation should include a history and physical examination to narrow the differential diagnosis[40]. When this examination does not identify another explanation for the pain, limited laboratory testing and transvaginal ultrasound scanning is often employed to rule out organic disease and reassure the patient[40]. The laboratory workup should include: complete blood count, beta human chorionic gonadotropin level, erythrocyte sedimentation rate, vaginal swabs for Chlamydia and Gonorrhea, and urinalysis with urine and culture[40]. Additional magnetic resonance imaging should be considered when in doubt for organic disease and diagnostic laparoscopy may be eventually performed in selected cases[40]. A tense pelvic floor is often reported during vaginal examination in CPP and spasm of the pelvic floor muscles is considered a relevant etiologic factor[36]. In addition, up to 60% of patients may report symptoms of either voiding dysfunction or dyschezia[42].

Treatment

Physiotherapy to relax the pelvic floor is often prescribed as first-line treatment for CPP, but randomized, controlled trials to confirm its effectiveness are lacking. Vaginal electrical stimulation was retrospectively reported to decrease pain in 52% of 66 chronic pelvic pain patients when coexistent levator ani spasm was also diagnosed by clinical exam[43]. Benefits were generally sustained during a 30-wk follow-up. Nonetheless, few studies have evalu-

ated pelvic floor function in women with CPP. Abbott and coworkers reported pelvic floor myalgia in 68 out of 118 patients referred for long-standing, unresponsive CPP[44]. They diagnosed pelvic floor myalgia based on objective evidence of contracted, painful pelvic muscles on palpation and elevated resting intraluminal pressures as measured by vaginal manometry. This study was a double blind, placebo controlled trial to test the efficacy of BoTox A in patients who had CPP with pelvic floor spasm. BoTox injection was associated with a significant reduction in vaginal resting pressure compared to placebo, but pain was only partially relieved and was not significantly different between BoTox and placebo. The authors concluded that pelvic floor spasm can cause CPP and that improvement in some symptoms occurs following reductions in muscle spasm[44]. In open studies, a number of treatment modalities have been reported to be effective for decreasing symptoms in CPP. These options include either oral or intramuscular hormone therapy, levator ani trigger point steroid injections, and sacral neuromodulation[36,40,42,45]. Most studies are open, retrospective, single center experiences with poor generalizability of treatment outcome.

Tricyclic antidepressants and Sertraline seem to work no better than placebo in CPP[40]. Surgery should be limited to patients with an organic cause for pelvic pain[36,40]. In non-responsive, severely disabled patients a multidisciplinary approach is advocated to fit with a biopsychosocial model of pain[40].

CONCLUSION

Chronic anorectal and pelvic pain syndromes receive little research attention despite the fact that they are prevalent, often disabling pain syndromes which are associated with significant health care costs and quality of life impact. Their frequency in the general population may be as high as 24% for chronic pelvic pain in women and 6.6% for chronic proctalgia. It is common for these patients to be referred to multiple specialists. Etiology is poorly defined, but chronic tension (spasm) in the striated muscles of the pelvic floor is often considered to be the pathophysiological mechanism for most of them. A recent randomized, controlled trial provided evidence that dyssynergic defecation (i.e., paradoxical contraction or failure to relax the pelvic floor muscles when straining to defecate) is the primary cause of pain for the majority of patients with chronic proctalgia, even for patients who do not complain of constipation. Biofeedback to treat dyssynergic defecation was an effective treatment for the subset of patients with chronic proctalgia who reported tenderness when traction was applied to the levator ani muscles during digital rectal exam (a sign of excessive tension in these muscles). This finding should prompt researchers to look for features of dyssynergic defecation in other pelvic pain syndromes and to try a similar treatment. A multidisciplinary and tailored approach to treat anorectal and pelvic pain patients without pelvic floor dysfunction is strongly suggested.

REFERENCES

1 **Drossman DA**, Li Z, Andruzzi E, Temple RD, Talley NJ, Thompson WG, Whitehead WE, Janssens J, Funch-Jensen P, Corazziari E. U.S. householder survey of functional gastrointestinal disorders. Prevalence, sociodemography, and health impact. *Dig Dis Sci* 1993; **38**: 1569-1580

2 **Chiarioni G**, Nardo A, Vantini I, Romito A, Whitehead WE. Biofeedback is superior to electrogalvanic stimulation and massage for treatment of levator ani syndrome. *Gastroenterology* 2010; **138**: 1321-1329

3 **Wald A,** Bharucha AE, Enck P, Rao SSC. Functional anorectal disorders. 3rd ed. Drossman DA, Corazzairi E, Delvaux M, Spiller RC, Talley NJ, Thompson WG, and Whitehead WE, editors. Rome III: The Functional Gastrointestinal Disorders. McLean: Degnon Associates, 2006: 639-685

4 **Thiele Gh**. Coccygodynia: cause and treatment. *Dis Colon Rectum* 1963; **6**: 422-436

5 **Whitehead WE,** Wald A, Diamant NE, Enck P, Pemberton JH, and Eao SSC. Functional disorders of the anus and rectum. 2nd ed. Drossman DA, Corazziari E, Talley NJ, Thompson WG, and Whitehead WE, editors. Rome II: The Functional Gastrointestinal Disorders. McLean: Degnon Associates, 2000: 483-542

6 **Gilliland R**, Heymen JS, Altomare DF, Vickers D, Wexner SD. Biofeedback for intractable rectal pain: outcome and predictors of success. *Dis Colon Rectum* 1997; **40**: 190-196

7 **Wald A**. Functional anorectal and pelvic pain. *Gastroenterol Clin North Am* 2001; **30**: 243-51, viii-ix

8 **Park DH**, Yoon SG, Kim KU, Hwang DY, Kim HS, Lee JK, Kim KY. Comparison study between electrogalvanic stimulation and local injection therapy in levator ani syndrome. *Int J Colorectal Dis* 2005; **20**: 272-276

9 **Salvati EP**. The levator syndrome and its variant. *Gastroenterol Clin North Am* 1987; **16**: 71-78

10 **Renzi C**, Pescatori M. Psychologic aspects in proctalgia. *Dis Colon Rectum* 2000; **43**: 535-539

11 **Ger GC**, Wexner SD, Jorge JM, Lee E, Amaranath LA, Heymen S, Nogueras JJ, Jagelman DG. Evaluation and treatment of chronic intractable rectal pain--a frustrating endeavor. *Dis Colon Rectum* 1993; **36**: 139-145

12 **Christiansen J**, Bruun E, Skjoldbye B, Hagen K. Chronic idiopathic anal pain: analysis of ultrasonography, pathology, and treatment. *Dis Colon Rectum* 2001; **44**: 661-665

13 **Lanzara A**. [Vincenzo Monaldi, university teacher]. *Arch Monaldi Mal Torace* 1991; **46**: 183-188

14 **Hompes R**, Jones OM, Cunningham C, Lindsey I. What causes chronic idiopathic perineal pain? *Colorectal Dis* 2011; **13**: 1035-1039

15 **Grant SR**, Salvati EP, Rubin RJ. Levator syndrome: an analysis of 316 cases. *Dis Colon Rectum* 1975; **18**: 161-163

16 **Sohn N**, Weinstein MA, Robbins RD. The levator syndrome and its treatment with high-voltage electrogalvanic stimulation. *Am J Surg* 1982; **144**: 580-582

17 **Hull TL**, Milsom JW, Church J, Oakley J, Lavery I, Fazio V. Electrogalvanic stimulation for levator syndrome: how effective is it in the long-term? *Dis Colon Rectum* 1993; **36**: 731-733

18 **Billingham RP**, Isler JT, Friend WG, Hostetler J. Treatment of levator syndrome using high-voltage electrogalvanic stimulation. *Dis Colon Rectum* 1987; **30**: 584-587

19 **Heah SM**, Ho YH, Tan M, Leong AF. Biofeedback is effective treatment for levator ani syndrome. *Dis Colon Rectum* 1997; **40**: 187-189

20 **Rao SS**, Paulson J, Mata M, Zimmerman B. Clinical trial: effects of botulinum toxin on Levator ani syndrome--a double-blind, placebo-controlled study. *Aliment Pharmacol Ther* 2009; **29**: 985-991

21 **Falletto E**, Masin A, Lolli P, Villani R, Ganio E, Ripetti V, Infantino A, Stazi A. Is sacral nerve stimulation an effective

treatment for chronic idiopathic anal pain? *Dis Colon Rectum* 2009; **52**: 456-462
22 **Mayer EA**, Aziz Q, Coen S, Kern M, Labus JS, Lane R, Kuo B, Naliboff B, Tracey I. Brain imaging approaches to the study of functional GI disorders: a Rome working team report. *Neurogastroenterol Motil* 2009; **21**: 579-596
23 **Mazza L**, Formento E, Fonda G. Anorectal and perineal pain: new pathophysiological hypothesis. *Tech Coloproctol* 2004; **8**: 77-83
24 **Andromanakos NP**, Kouraklis G, Alkiviadis K. Chronic perineal pain: current pathophysiological aspects, diagnostic approaches and treatment. *Eur J Gastroenterol Hepatol* 2011; **23**: 2-7
25 **Traycoff RB**, Crayton H, Dodson R. Sacrococcygeal pain syndromes: diagnosis and treatment. *Orthopedics* 1989; **12**: 1373-1377
26 **Maigne JY**, Lagauche D, Doursounian L. Instability of the coccyx in coccydynia. *J Bone Joint Surg Br* 2000; **82**: 1038-1041
27 **Maigne JY**, Doursounian L, Chatellier G. Causes and mechanisms of common coccydynia: role of body mass index and coccygeal trauma. *Spine (Phila Pa 1976)* 2000; **25**: 3072-3079
28 **Takano M**. Proctalgia fugax: caused by pudendal neuropathy? *Dis Colon Rectum* 2005; **48**: 114-120
29 **Wray CC**, Easom S, Hoskinson J. Coccydynia. Aetiology and treatment. *J Bone Joint Surg Br* 1991; **73**: 335-338
30 **Maigne JY**, Chatellier G, Faou ML, Archambeau M. The treatment of chronic coccydynia with intrarectal manipulation: a randomized controlled study. *Spine (Phila Pa 1976)* 2006; **31**: E621-E627
31 **Cebesoy O**, Guclu B, Kose KC, Basarir K, Guner D, Us AK. Coccygectomy for coccygodynia: do we really have to wait? *Injury* 2007; **38**: 1183-1188
32 **Karadimas EJ**, Trypsiannis G, Giannoudis PV. Surgical treatment of coccygodynia: an analytic review of the literature. *Eur Spine J* 2011; **20**: 698-705
33 **Benson JT**, Griffis K. Pudendal neuralgia, a severe pain syndrome. *Am J Obstet Gynecol* 2005; **192**: 1663-1668
34 **Robert R**, Prat-Pradal D, Labat JJ, Bensignor M, Raoul S, Rebai R, Leborgne J. Anatomic basis of chronic perineal pain: role of the pudendal nerve. *Surg Radiol Anat* 1998; **20**: 93-98
35 **Labat JJ**, Riant T, Robert R, Amarenco G, Lefaucheur JP, Rigaud J. Diagnostic criteria for pudendal neuralgia by pudendal nerve entrapment (Nantes criteria). *Neurourol Urodyn* 2008; **27**: 306-310
36 **Dalpiaz O**, Kerschbaumer A, Mitterberger M, Pinggera G, Bartsch G, Strasser H. Chronic pelvic pain in women: still a challenge. *BJU Int* 2008; **102**: 1061-1065
37 **Mathias SD**, Kuppermann M, Liberman RF, Lipschutz RC, Steege JF. Chronic pelvic pain: prevalence, health-related quality of life, and economic correlates. *Obstet Gynecol* 1996; **87**: 321-327
38 **Zondervan KT**, Yudkin PL, Vessey MP, Jenkinson CP, Dawes MG, Barlow DH, Kennedy SH. The community prevalence of chronic pelvic pain in women and associated illness behaviour. *Br J Gen Pract* 2001; **51**: 541-547
39 **Gelbaya TA**, El-Halwagy HE. Focus on primary care: chronic pelvic pain in women. *Obstet Gynecol Surv* 2001; **56**: 757-764
40 **Daniels JP**, Khan KS. Chronic pelvic pain in women. *BMJ* 2010; **341**: c4834
41 **Matheis A**, Martens U, Kruse J, Enck P. Irritable bowel syndrome and chronic pelvic pain: a singular or two different clinical syndrome? *World J Gastroenterol* 2007; **13**: 3446-3455
42 **Everaert K**, Devulder J, De Muynck M, Stockman S, Depaepe H, De Looze D, Van Buyten J, Oosterlinck W. The pain cycle: implications for the diagnosis and treatment of pelvic pain syndromes. *Int Urogynecol J Pelvic Floor Dysfunct* 2001; **12**: 9-14
43 **Fitzwater JB**, Kuehl TJ, Schrier JJ. Electrical stimulation in the treatment of pelvic pain due to levator ani spasm. *J Reprod Med* 2003; **48**: 573-577
44 **Abbott JA**, Jarvis SK, Lyons SD, Thomson A, Vancaille TG. Botulinum toxin type A for chronic pain and pelvic floor spasm in women: a randomized controlled trial. *Obstet Gynecol* 2006; **108**: 915-923
45 **Langford CF**, Udvari Nagy S, Ghoniem GM. Levator ani trigger point injections: An underutilized treatment for chronic pelvic pain. *Neurourol Urodyn* 2007; **26**: 59-62

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EDITORIAL

Multiphoton microscopy: An introduction to gastroenterologists

Hye Jin Cho, Hoon Jai Chun, Eun Sun Kim, Bong Rae Cho

Hye Jin Cho, Hoon Jai Chun, Eun Sun Kim, Institute of Digestive Disease and Nutrition, Department of Internal Medicine, Korea University College of Medicine, 136-705 Seoul, South Korea
Bong Rae Cho, Department of Chemistry, Korea University, 136-705 Seoul, South Korea
Author contributions: Cho HJ, Chun HJ, Kim ES designed research and performed research; Cho BR contributed new reagents; Cho HJ analyzed data and wrote the paper.
Correspondence to: Hoon Jai Chun, MD, PhD, AGAF, Professor, Institute of Digestive Disease and Nutrition, Department of Internal Medicine, Korea University College of Medicine, 136-705 Seoul, South Korea. drchunhj@chol.com
Telephone: +82-2-9206555 Fax: +82-2-9531943
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Abstract

Multiphoton microscopy, relying on the simultaneous absorption of two or more photons by a fluorophore, has come to occupy a prominent place in modern biomedical research with its ability to allow real-time observation of a single cell and molecules in intact tissues. Multiphoton microscopy exhibits nonlinear optical contrast properties, which can make it possible to provide an exceptionally large depth penetration with less phototoxicity. This system becomes more and more an inspiring tool for a non-invasive imaging system to realize "optical biopsy" and to examine the functions of living cells. In this review, we briefly present the physical principles and properties of multiphoton microscopy as well as the current applications in biological fields. In addition, we address what we see as the future potential of multiphoton microscopy for gastroenterologic research.

Key words: Multiphoton microscopy; Optical biopsy; Gastrointestinal disease

Peer reviewer: Kevin J Spring, Dr., PhD, Conjoint Gastroenterology Laboratory, The Queensland Institute of Medical Research, the Bancroft Centre, Rm H07, PO Royal Brisbane Hospital, Herston, QLD 4029, Australia

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INTRODUCTION

A remarkable evolution of biological science has induced the need to visualize cells in an intact whole organism. To date, most applications using microscopy are limited to fixed cells or excised tissues. However, characterization of morphological features and establishment of cell function of native tissues is important for the diagnosis of disease in the early stage and has improved understanding of the associated pathophysiological processes. Therefore, the need for real-time observation of cells and their subcellular components in intact tissues is of great interest and imaging techniques have been developed to pursue this goal.

One of these techniques is fluorescence imaging. Although the combination of microscopy with fluorescent labeling has improved sensitivity, this approach provides only a two-dimensional view of samples. The use of confocal microscopy allows for the observation of subcellular material with three-dimensional resolution. However, confocal microscopy is limited by the effective imaging depth of typically less than 100 μm and phototoxicity, which is caused by using a short wavelength laser[1].

Recent advances in nonlinear optical processes of multiphoton microscopy compensate single photon-linear microscopy technologies such as confocal microscopy by the capacity for deeper tissue penetration with

clear images and the reduction of direct ultraviolet damage[2]. Thus, multiphoton microscopy has been applied to various parts of the imaging task and has now become the technique of choice for subcellular observations of thick tissues and in living animals[3].

In addition, endoscopists often want to know the relationship between the gross endoscopic findings and the microscopic diagnosis during routine endoscopy. Although a mucosal biopsy is the standard method for histopathological diagnosis of an abnormal mucosal lesion, this approach is limited by sampling error, bleeding risk and the time lag for results. Therefore, endoscopists would like to have the ability to directly observe and promptly identify pathology of cellular and/or subcellular structures without biopsy. Multiphoton microscopy has the full potential to achieve this goal because it can provide thin optical sections from thick specimens.

In this article, the principles of multiphoton microscopy and its applications in bioscience are reviewed, as well as the prospects for clinical use.

MULTIPHOTON MICROSCOPY

Early in the development of quantum mechanics, the theoretical concept was first proposed by Göppert-Mayer in 1931. Multiphoton excitation is based on the probability that fluorophore molecules are excited by multiple low energy photons that can arrive "simultaneously" at the fluorophore and interact with it. The fluorophore molecule absorbs the sum of the energy from each photon, and an electron in the fluorophore is transferred to the excited state, which can induce an electronic transition similar to a single high-energy photon[4]. Soon after, the molecule in the excited state falls back to the ground state with emission of fluorescence, which has most, but not all, of the initial energy, owing to non-radiative relaxation (Figure 1).

Because the energy of a photon is inversely proportional to its wavelength (λ), the emitted fluorescence is a longer wavelength than the exciting light. However, in the case of multiphoton excitation, the fluorophore molecule almost simultaneously absorbs the energy from multiple photons, each of which contributes a part of the total energy required to induce the fluorescent emission. Thus, the emitted fluorescent photon has a shorter wavelength than each of the photons involved in excitation. For this reason, multiphoton microscopy can induce fluorescence equal to the energy of single photon excitation microscopy by low energy photons.

However, multiphoton excitation requires enormously high light intensities that, if continous, would almost instantly vaporize the specimen. Therefore, to generate enough fluorescence practical for multi-photon microscopy, a pulsed laser source is needed. In other words, using a laser that produces extremely brief pulses (femtosecond laser, about 10^{-5}) at a high repetition rate, thus generating high instantaneous energy but low average energy[5].

Multiphoton microscopy exhibits nonlinear optical contrast properties that are predicated upon second and third-order nonlinear interactions between light and particles[6]. The nonlinear optical effects are proportional to the square or cube of the fundamental light intensity; this gives multiphoton microscopy the intrinsic characteristics of 3-dimensional images. This is because the photon density is high at the focal point, and it falls off steeply from the focal point[7]. This eliminates out-of-focus contributions and allows multiphoton microscopy to obtain high resolution images from the scattered photons of the fluorophore emission used to produce the image. In addition, photobleaching is restricted to a narrow region around the plane of focus.

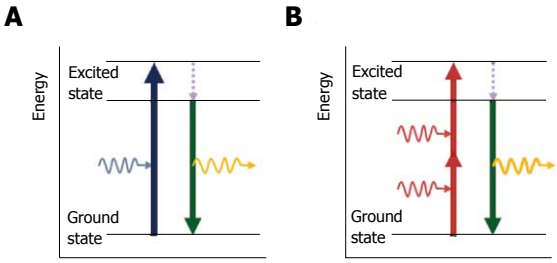

Figure 1 Multiphoton excitation. A: Single-photon excitation. Individual photons of high-energy blue light (wavelength, λ = 480 nm) excite fluorophores in the sample. After an electron in the fluorophore is transferred to the excited state (blue arrow), it loses energy rapidly owing to non-radiative relaxation (dashed arrow). Subsequently, fluorescence emission (yellow curved arrow) occurs at a longer wavelength than the excitation light as the electron falls back to the ground state (green arrow); B: Two-photon excitation. Two infrared photons (λ = 780 nm) are absorbed simultaneously (red arrows) to excite the fluorophore and light is emitted in the same manner as for single-photon excitation (green arrow) with emission of fluorescein.

To date, the most widely used imaging modalities associated with multiphoton microscopy are multiphoton excitation with fluorescence, second harmonic generation, multiphoton fluorescence lifetime imaging microscopy, and spectral lifetime imaging microscopy[1]. For example, with two-photon microscopy, a fluorophore molecule is excited by the nearly simultaneous absorption of two photons, each twice the wavelength required for a single photon excitation[8]. A molecule of fluorescein can be excited by two photons of near-infrared light (λ ≈ 780 nm), each of which has approximately half the energy of a single blue photon (λ ≈ 480 nm), and then emit a photon of green light, in the same manner as for standard (one-photon) excitation with blue light[7]. According to nonlinear excitation, fluorescent emission from fluorophore molecules is proportional to the square of the excitation intensity. This intensity-squared dependence of two-photon microscopy provides "optical sectioning" capability, without using an adjustable pinhole aperture in front of the detector to reject out-of-focus fluorescence like confocal microscopy[9].

Multiphoton microscopy has several advantages over confocal microscopy. Most of all, the use of long excitation wavelengths has major advantages. Since light scattering declines rapidly with an increasing wavelength, deeper penetration can be achieved by using a longer wavelength of light than with single-photon confocal microscopy. In addition to an increase in the penetration

Table 1 Comparison of two-photon microscopy with confocal microscopy

	Confocal microscopy	Two-photon microscopy
Excitation wavelength	Short (ultraviolet light)	Long (infra-red light)
Tissue imaging depth	About 50-100 μm	About 400-1000 μm
Spatial resolution	nm (3D-resolution with pinhole aperture)	nm (3D-resolution with inherent optical sectioning)
Photodamage and photobleaching	High	Low

depth, a longer wavelength of light, such as infrared light (700-1000 nm) used for multiphoton microscopy, has much less energy than confocal microscopy, and therefore causes negligible photodamage and phototoxicity to cells and tissues. Cells and molecules deep inside living tissues can be observed for long periods of time. Moreover, because excitation and emission take place only at the focal plane, multiphoton microscopy reduces the photobleaching outside of the focal plane, unlike confocal microscopy. Therefore, it results in high fluorescence collection efficiency and thus greater signal intensity at any given tissue depth.

Table 1 details comparative differences in excitation wavelength, tissue imaging depth, resolution and photo damage/bleaching between two-photon microscopy and confocal microscopy.

BIOLOGICAL APPLICATIONS

Multiphoton microscopy is a powerful tool for visualizing cellular and subcellular events within living tissue with its inherent "optical sectioning" capability, deeper penetration and minimal phototoxicity and photobleaching. Multiphoton microscopy can capture whole organisms or embryos on a large scale. Though transparent organisms such as the zebra fish and drosophila are ideal candidates for such studies, the development of the hamster embryo model has allowed for observations over long periods of time, for several days[10].

In addition to morphological studies, multiphoton microscopy can be used for dynamic and functional cellular imaging with the development of various fluorescent probes. For example, two-photon microscopy of the calcium sensitive fluorophore allows for the collection of subcellular spatial and temporal information on (Ca^{2+}) ion entry through voltage-gated channels or release from intracellular stores within a single myocyte at depths of up to 200 μm below the epicardial surface. Therefore, two-photon microscopy is well suited to determine the functional state of donor cells following intracardiac transplantation[11].

Neuroscientists use multiphoton microscopy for the observation of neuronal plastic changes within brain slices, measuring ionized-calcium dynamics deep in brain tissues[12]. The dendritic spines, which are a major functional component of the nervous system associated with learning and memory activated by chemical and electrical transmission mechanisms, are very tiny structures. Since neurons are very sensitive to phototoxicity and brain tissue is highly scattered, it has been difficult to visualize these dynamic processes in live tissues[13,14]. However, multiphoton microscopy overcomes these obstacles by using long wavelength light and providing high resolution deep imaging without causing injury to the living material[15]. It allows visualization of fine structures of the brain in the head and neck area, including unique signaling and dynamic motility of the dendritic spines 300-400 μm into the brain tissue[16,17].

Multiphoton microscopy enables imaging of dynamic and heterogeneous immune processes at the cellular and molecular levels deep within intact organs of living animals. Due to the depth of penetration and minimal photodamage, multiphoton microscopy permits six-dimensional (x, y, z, time, intensity, wavelength) imaging of intact lymphoid organs and can be used to observe naïve lymphocytes for hours without loss of viability or motility[18]. Dynamic movements and cellular interactions of viable T- and B-cells can be revealed, as well as the antigen presenting cells in the *in vivo* setting[7,19].

Multiphoton microscopy is also a preferred imaging technique for cancer research, for example in studies on angiogenesis and metastasis *in vivo*[20,21]. Tumor microinvasion and metastasis involves complex interactions between cells and extracellular matrix proteins, most notably collagen[22]. Due to the ability of imaging more deeply in tissues with less toxicity, multiphoton microscopy facilitates imaging of tumor-stroma interactions and thus facilitates improved understanding of the processes of cell migration, metastasis, and tumor progression with direct observation *in vivo*[23].

Gastrointestinal endoscopists have to rely on visual inspection for the diagnosis of disease. Therefore, multiphoton excitation imaging may be helpful in the diagnosis and offer additional diagnostic benefit. Indeed, a pilot study of multiphoton microscopy to diagnose gastric cancer has been reported recently[24]. The results of the study showed that multiphoton microscopy can be used to diagnose gastric cancer by optical biopsy. Multiphoton microscopy has proved to be a promising tool for real-time histological diagnosis. Recent developments in imaging technology now make this possible.

Multiphoton microscopy also has the ability to penetrate deeper inside the tissue and excite endogenous autofluorescence molecules such as intracellular nicotinamide adenine dinucleotide phosphate (reduced form), flavin, melanin and lipofuscin, instead of using fluorescent dyes which must be used for *in vivo* confocal laser microscopy[25]. It provides the ability to detect cellular and subcellular details of the gastrointestinal mucosa without fixation or staining. Multiphoton imaging of intact human gastrointestinal mucosa *ex vivo* provides improved cellular detail compared to confocal imaging, without the need for fluorescent dyes[2].

Suitable indicators for two-photon microscopy are required in order to get a clearer image. Recently, our col-

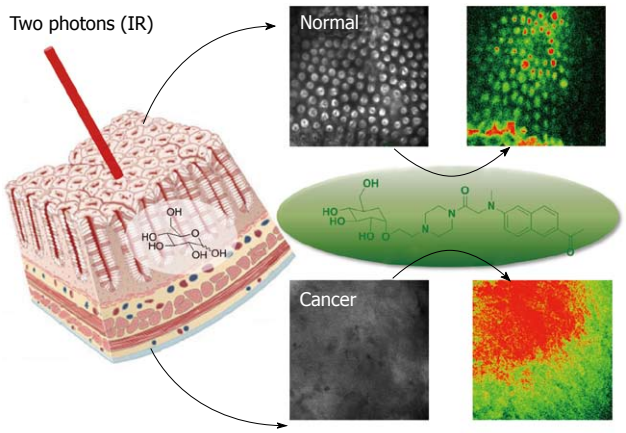

Figure 2 Images of normal tissue (above) and cancer tissue (below) treated with AG2. Normal tissues were incubated in artificial cerebrospinal fluid (ACSF) for 4 h, and cancer tissues were incubated in ACSF for 4 h, after which AG2 uptake was monitored. Right-side images are bright-field images, left-side images are pseudocolored two-photon microscopy (TPM) images obtained after incubation with AG2 for 4 h. The TPM images were obtained at a depth of 100 μm by collecting the two-photon excited fluorescence spectra in the range of 520-620 nm on excitation with fs pulses at 780 nm. IR: Infrared.

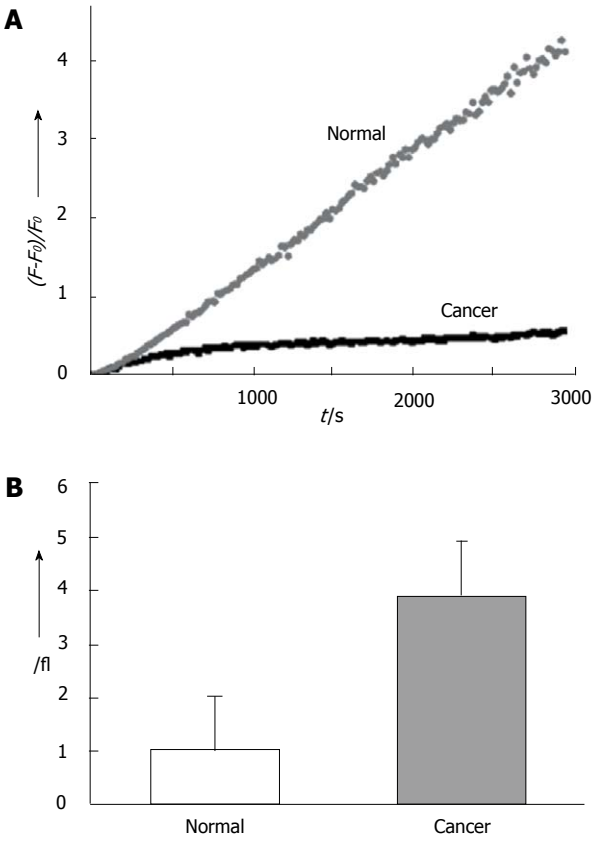

Figure 3 AG2 uptake in normal and cancerous tissues. A: Time course of AG2 uptake by normal tissue and cancer tissue at 100 μm depth as a function of time; B: Relative AG2 uptake by normal tissue and cancer tissue for 4000 s. The columns indicate the sum of the two-photon excited fluorescence intensities measured by photomultiplier tube at depths of 75, 100, 125 and 150 μm from the tissue surface, relative to that of normal tissue. The data are the average of three independent experiments.

laborators have developed many new two-photon tracers. One tracer, a hydrogen probe, AH2, which emits fluorescence at pH < 4 can be used to obtain images of live esophageal tissue from the mucosal surface to 100 μm in depth. Emitted fluorescence of the hydrogen probe in reflux esophagitis tissue was stronger than that in control tissue. Multiphoton-emitted fluorescence of low esophageal tissue of the reflux model was similar to that of stomach[26]. Visible images of pH changes in reflux esophageal tissue can be obtained by use of the multiphoton hydrogen probe.

Another new probe, AG2, that can be easily taken up by cancer cells and tissues through glucose-specific translocation has been developed. AG2 shows negligible cytotoxicity and high photostability. It can monitor glucose uptake in colon cancer tissues and visualize at depth of 75-150 μm by two-photon microscopy (Figure 2). This compound may be useful in diagnosing the early stages of cancer and make it possible to develop customized cancer therapy according to the uptake rates of AG2 in normal and cancerous tissues (Figure 3). In addition, this laboratory has used multiphoton laser scanning microscopy to study gastric and colon cancer with other probes. Multiphoton images of normal and cancer cell lines, as well as normal mucosa and dysplastic tissues, (adenoma, adenocarcinoma) labeled with the multiphoton microscopy probes AZn1 and ACu1, have been studied. The findings showed that the Cu1 content was higher, Zn1 content was lower, and the ratio of Cu1 to Zn1 was much higher in adenomas and adenocarcinoma than in the normal mucosa. These results suggest the possibility that multiphoton endomicroscopy might be developed further to use as a technique for performing virtual biopsies during the course of routine endoscopy.

CONCLUSION

Multiphoton microscopy has rapidly evolved and become a standard device for cell-based biological research in the fields of genomics, proteomics and tissue engineering. A major advantage of multiphoton microscopy is the ability to observe deep within intact organs and cells. Its applications are being extended beyond basic research to the clinical setting, such as detection of skin cancers, mucosal dysplasia of the intestinal tract, Alzheimer's disease, and metabolic disorders just by visualizing patient's tissue at the cellular level of resolution[27].

Although multiphoton microscopy has already been used by many biologists for research and some clinicians, as mentioned briefly above, its advantages are partly limited by the bulkiness of the system including lasers, objective lenses, and scanning devices. Therefore, several groups currently are trying to develop smaller fluorescence microscopes, either by using a gradient index lens as a thin, rodlike probe to extend the working distance of a conventional objective[15] or by using fiber optics to construct multiphoton endoscopes[28]. Imaging of goblet cells as a marker for intestinal metaplasia of the stomach

by two-photon endomicroscopy has been reported[29]. Its techniques can three-dimensionally observe goblet cells in mouse large intestine, and it provides the possibility that two photon endomicroscopy is advantageous in diagnoses.

The development of miniature laser scanning multiphoton endoscopes will provide advantages over currently available endomicroscopy technologies and be of great utility to gastroenterologists. Moreover, miniature multiphoton endoscopy may be used for minimally invasive endoscopic procedures and has enormous potential for histological evaluation of organs outside the gastrointestinal tract, namely, the liver, pancreas, and ovaries by transluminal endoscopic approaches[30].

With the development of novel laser sources, new fluorophores and more specific probes, multiphoton microscopy and its applications will open up a wide range of possibilities. In addition, it can be combined with other imaging modalities such as ultrasound or magnetic resonance imaging, which provide complementary information.

The development of multiphoton microscopy marks a significant step in the advancement of imaging modalities and will likely aid in our understanding of the basis of disease as well as the management of the clinical manifestations of disease.

REFERENCES

1 **Provenzano PP**, Eliceiri KW, Keely PJ. Multiphoton microscopy and fluorescence lifetime imaging microscopy (FLIM) to monitor metastasis and the tumor microenvironment. *Clin Exp Metastasis* 2009; **26**: 357-370

2 **Rogart JN**, Nagata J, Loeser CS, Roorda RD, Aslanian H, Robert ME, Zipfel WR, Nathanson MH. Multiphoton imaging can be used for microscopic examination of intact human gastrointestinal mucosa ex vivo. *Clin Gastroenterol Hepatol* 2008; **6**: 95-101

3 **Zipfel WR**, Williams RM, Webb WW. Nonlinear magic: multiphoton microscopy in the biosciences. *Nat Biotechnol* 2003; **21**: 1369-1377

4 **Williams RM**, Zipfel WR, Webb WW. Multiphoton microscopy in biological research. *Curr Opin Chem Biol* 2001; **5**: 603-608

5 **Wang BG**, König K, Halbhuber KJ. Two-photon microscopy of deep intravital tissues and its merits in clinical research. *J Microsc* 2010; **238**: 1-20

6 **Carriles R**, Schafer DN, Sheetz KE, Field JJ, Cisek R, Barzda V, Sylvester AW, Squier JA. Invited review article: Imaging techniques for harmonic and multiphoton absorption fluorescence microscopy. *Rev Sci Instrum* 2009; **80**: 081101

7 **Cahalan MD**, Parker I, Wei SH, Miller MJ. Two-photon tissue imaging: seeing the immune system in a fresh light. *Nat Rev Immunol* 2002; **2**: 872-880

8 **Stutzmann GE**, Parker I. Dynamic multiphoton imaging: a live view from cells to systems. *Physiology* (Bethesda) 2005; **20**: 15-21

9 **Webb RH**. Theoretical basis of confocal microscopy. *Methods Enzymol* 1999; **307**: 3-20

10 **Squirrell JM**, Wokosin DL, White JG, Bavister BD. Long-term two-photon fluorescence imaging of mammalian embryos without compromising viability. *Nat Biotechnol* 1999; **17**: 763-767

11 **Rubart M**. Two-photon microscopy of cells and tissue. *Circ Res* 2004; **95**: 1154-1166

12 **Helmchen F**, Waters J. Ca2+ imaging in the mammalian brain in vivo. *Eur J Pharmacol* 2002; **447**: 119-129

13 **Jung JC**, Mehta AD, Aksay E, Stepnoski R, Schnitzer MJ. In vivo mammalian brain imaging using one- and two-photon fluorescence microendoscopy. *J Neurophysiol* 2004; **92**: 3121-3133

14 **Oheim M**, Beaurepaire E, Chaigneau E, Mertz J, Charpak S. Two-photon microscopy in brain tissue: parameters influencing the imaging depth. *J Neurosci Methods* 2001; **111**: 29-37

15 **Levene MJ**, Dombeck DA, Kasischke KA, Molloy RP, Webb WW. In vivo multiphoton microscopy of deep brain tissue. *J Neurophysiol* 2004; **91**: 1908-1912

16 **Holthoff K**, Tsay D, Yuste R. Calcium dynamics of spines depend on their dendritic location. *Neuron* 2002; **33**: 425-437

17 **Majewska A**, Tashiro A, Yuste R. Regulation of spine calcium dynamics by rapid spine motility. *J Neurosci* 2000; **20**: 8262-8268

18 **Miller MJ**, Wei SH, Parker I, Cahalan MD. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* 2002; **296**: 1869-1873

19 **Meyer-Hermann ME**, Maini PK. Interpreting two-photon imaging data of lymphocyte motility. *Phys Rev E Stat Nonlin Soft Matter Phys* 2005; **71**: 061912

20 **Brown EB**, Campbell RB, Tsuzuki Y, Xu L, Carmeliet P, Fukumura D, Jain RK. In vivo measurement of gene expression, angiogenesis and physiological function in tumors using multiphoton laser scanning microscopy. *Nat Med* 2001; **7**: 864-868

21 **McDonald DM**, Choyke PL. Imaging of angiogenesis: from microscope to clinic. *Nat Med* 2003; **9**: 713-725

22 **Provenzano PP**, Eliceiri KW, Campbell JM, Inman DR, White JG, Keely PJ. Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC Med* 2006; **4**: 38

23 **Brown E**, McKee T, diTomaso E, Pluen A, Seed B, Boucher Y, Jain RK. Dynamic imaging of collagen and its modulation in tumors in vivo using second-harmonic generation. *Nat Med* 2003; **9**: 796-800

24 **Yan J**, Chen G, Chen J, Liu N, Zhuo S, Yu H, Ying M. A pilot study of using multiphoton microscopy to diagnose gastric cancer. *Surg Endosc* 2011; **25**: 1425-1430

25 **Hoffman A**, Goetz M, Vieth M, Galle PR, Neurath MF, Kiesslich R. Confocal laser endomicroscopy: technical status and current indications. *Endoscopy* 2006; **38**: 1275-1283

26 **Tian YS**, Lee HY, Lim CS, Park J, Kim HM, Shin YN, Kim ES, Jeon HJ, Park SB, Cho BR. A two-photon tracer for glucose uptake. *Angew Chem Int Ed Engl* 2009; **48**: 8027-8031

27 **Zipfel WR**, Williams RM, Christie R, Nikitin AY, Hyman BT, Webb WW. Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation. *Proc Natl Acad Sci USA* 2003; **100**: 7075-7080

28 **Helmchen F**. Miniaturization of fluorescence microscopes using fibre optics. *Exp Physiol* 2002; **87**: 737-745

29 **Bao H**, Boussioutas A, Reynolds J, Russell S, Gu M. Imaging of goblet cells as a marker for intestinal metaplasia of the stomach by one-photon and two-photon fluorescence endomicroscopy. *J Biomed Opt* 2009; **14**: 064031

30 **Kalloo AN**, Singh VK, Jagannath SB, Niiyama H, Hill SL, Vaughn CA, Magee CA, Kantsevoy SV. Flexible transgastric peritoneoscopy: a novel approach to diagnostic and therapeutic interventions in the peritoneal cavity. *Gastrointest Endosc* 2004; **60**: 114-117

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REVIEW

Can intraluminal devices prevent or reduce colorectal anastomotic leakage: A review

Annelien N Morks, Klaas Havenga, Rutger J Ploeg

Annelien N Morks, Klaas Havenga, Rutger J Ploeg, Division of Abdominal Surgery, Department of Surgery, University Medical Center Groningen, 9700 RB Groningen, The Netherlands
Author contributions: Morks AN wrote the paper; Havenga K and Ploeg RJ both substantially contributed to the design of the review, revised the review, and approved the final version.
Correspondence to: Klaas Havenga, MD, PhD, Division of Abdominal Surgery, Department of Surgery, University Medical Center Groningen, BA 11, Hanzeplein 1, 9700 RB Groningen, The Netherlands. k.havenga@umcg.nl
Telephone: +31-50-3612283 Fax: +31-50-3614873
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Peer reviewer: Omar Vergara-Fernandez, MD, Departments of Surgery, National Institute for Medical Sciences and Nutrition Salvador Zubirán, Vasco de Quiroga No. 15, Col Seccion XVI Deleg Tlalpan, CP 14000, México

Morks AN, Havenga K, Ploeg RJ. Can intraluminal devices prevent or reduce colorectal anastomotic leakage: A review. *World J Gastroenterol* 2011; 17(40): 4461-4469 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i40/4461.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i40.4461

Abstract

Colorectal anastomotic leakage is a serious complication of colorectal surgery, leading to high morbidity and mortality rates. In recent decades, many strategies aimed at lowering the incidence of anastomotic leakage have been examined. The focus of this review will be on mechanical aids protecting the colonic anastomosis against leakage. A literature search was performed using MEDLINE, EMBASE, and The Cochrane Collaborative library for all papers related to prevention of anastomotic leakage by placement of a device in the colon. Devices were categorised as decompression devices, intracolonic devices, and biodegradable devices. A decompression device functions by keeping the anal sphincter open, thereby lowering the intraluminal pressure and lowering the pressure on the anastomosis. Intracolonic devices do not prevent the formation of dehiscence. However, they prevent the faecal load from contacting the anastomotic site, thereby preventing leakage of faeces into the peritoneal cavity. Many attempts have been made to find a device that decreases the incidence of AL; however, to date, none of the devices have been widely accepted.

INTRODUCTION

Colorectal anastomotic leakage (AL) is a serious complication after colorectal surgery and may lead to high morbidity and mortality rates. The incidence of AL varies between 2.5% and 20% and the aetiology is multifactorial[1-5].

In recent decades, many strategies aimed at lowering the incidence of anastomotic leakage have been examined. A protective stoma reduces the consequence of anastomotic failure, thereby preventing the number of clinical leaks[6,7]. The Dutch TME trial demonstrated a lower rate of surgical re-intervention in patients with a diverting stoma ($P < 0.001$)[2]. However, a protective stoma can also result in stoma-related complications, and the obligatory operation to take down the stoma is associated with additional mortality, morbidity, and cost[8]. In addition, so-called temporary protective stomas tend to be left *in situ* for much longer than initially anticipated, sometimes even lifelong[9].

Many definitions are used to describe anastomotic leakage. The Surgical infection Study Group (1991) categorised AL into clinical and subclinical leakage[10]. In 2001, Bruce *et al*[11] recommended the subdivision of anastomotic leakage into three groups: radiological (no

clinical signs), clinical minor (no intervention needed), and clinical major (intervention required) leakage. This grading of AL resembles the grading of AL proposed by the International Study Group of Rectal Cancer (ISREC)[12]. The ISREC defines AL as a communication between the intra- and extraluminal compartments due to a defect of the integrity of the intestinal wall at the anastomosis between the colon and rectum or the colon and anus. The extent or severity of AL should be graded according to the impact on clinical management. Grade A does not require active therapeutic intervention; grade B requires active therapeutic intervention, but is manageable without relaparotomy; and grade C requires relaparotomy.

In this review, we focus on the use and potential success of mechanical intraluminal devices that may protect a colonic anastomosis against leakage. Different strategies have been adopted to lower the incidence of AL. In this respect we will differentiate between transanal decompression, intracolonic, and biodegradable devices. In addition, devices encircling the bowel have been tested. The use of some of these devices showed promising results in lowering the incidence of AL (Table 1).

Considering the persisting associated morbidity and mortality of AL, and availability of intraluminal colonic devices today, a revival of the discussion of their effectiveness in lowering the incidence of AL is worthwhile.

LITERATURE SEARCH

This is a retrospective review describing the literature on devices protecting colonic anastomoses. In March 2010, an extensive literature search was performed using MEDLINE, EMBASE, and The Cochrane Collaborative Library for all papers related to prevention of anastomotic leakage by placement of an intraluminal device in the colon. Our search comprised the following: (tube OR tubes OR bypass* OR by-pass OR stent* OR device* OR coloshield) AND (anastomosis OR anastomo*) AND (leak* OR dehiscen*) AND (colon OR rectum OR colonic OR intracolonic OR colorectal) AND (prevent* OR protect*). Articles were marked as relevant if an intraluminal device was studied that protected a colonic anastomosis from leakage. Articles describing glues or fluids that protect the anastomosis are not included in this review, neither are studies on techniques of anastomosing the bowel. The reference list of each relevant article was checked for further relevant papers. All first authors of relevant papers were checked for other relevant publications. All articles were selected by one reviewer and in case of doubt, a second reviewer was consulted. The Internet was also searched using www.scholar.google.com. The search yielded 337 articles of which 44 were related to an intraluminal device intended to protect a colonic anastomosis. These 44 articles include experimental animal studies, as well as retrospective and prospective clinical studies.

TRANSANAL DECOMPRESSION DEVICES

A decompression device functions by keeping the anal sphincter open, thereby decreasing the intraluminal pressure, as well as the pressure on the anastomosis. In this way, the device serves as a protective vent. In addition, a number of authors have hypothesized that some tubes permit reinforcement and prevent angulation of the bowel and anastomosis. As early as in the 13th century, Lanfrank reported the placement of a reed pipe as an intraluminal stent in the colon[13]. More recently, Gurjar[14] assessed the current practice of rectal tubes in the United Kingdom and Ireland. A questionnaire was sent to all members of the Association of Coloproctology (ACPGBI). The response rate was 58%, and 35% of those reported to use a rectal tube, in the majority of cases after ileo-anal or colonic pouch surgery. Sixteen percent used the tube after low anterior resection (LAR). Predominantly, a Foley catheter was positioned above the anastomosis (80%). The catheter was left *in situ* for a median of five days. Most respondents used the tube with the intention to decompress the rectum and/or pouch.

Animal studies

In 1988, Goldman *et al*[15] tested an intrarectal, conically shaped flexible silastic tube in a dog model of LAR. The tube was fixed to the submucosa 5 cm proximal to the anastomosis. Twenty-five dogs underwent an LAR; 15 with a tube and 10 controls. In some animals, the anastomosis was deliberately made incomplete, leaving gaps. Mortality occurred only in the control group. Morbidity in the control group was six times higher (three colocutaneous fistulae and three anastomotic abscesses). Only one dog with a tube and an incomplete anastomosis was diagnosed with a pelvic abscess. The tubal fixation sites showed oedema and minor inflammatory reaction on microscopic examination. The authors concluded that their procedure presented effective practical implications, such as omitting the need for a proximal protective colostomy.

Human studies

Indwelling rectal tubes: Stewart[16] used an indwelling rectal tube in 153 patients who underwent a left hemicolectomy or sigmoid resection. After completion of the anastomosis, a No. 32-34 French latex tube was introduced through the anal canal and directed through the anastomosis to a distance of approximately 15 centimeters above the anastomosis. The rectal tube was sutured to the perianal skin for fixation. Twice daily, the tube was irrigated with neomycin solution and after five or six days, the tube was removed. Suture-line complications occurred in seven patients (4.6%), with three patients being graded as C according to the ISREC classification. In the other four patients, the anastomotic complications were grade A/B (haematoma, stricture, and abscess noted only on sigmoidoscopy). Adverse effects of the tube (e.g., ulceration of the colon) were not observed.

Table 1 Studies on intracolonic devices aimed at preventing anastomotic leakage

Study[Ref.]	Yr	*n*	Site	Device	Anastomotic complications
Rack[17]	1966	32	Sigmoid or rectum resection	Rectal tube	0 AL
Stewart *et al*[16]	1968	153	Left colon or colorectal resection	Rectal tube	4 Grade A/B AL (3%) 3 Grade C AL (2%)
Balz *et al*[18]	1978	392 (including 153 patients from study Stewart)	Left colon or colorectal resection	Rectal tube	3 Grade A/B AL (1%) 6 Grade C AL (2%)
Castrini *et al*[33]	1984	19	Left colon or rectal resection	Intracolonic bypass	0 AL
Ravo *et al*[35]	1987	28	Sigmoid resection	Intracolonic bypass	0 AL
Cuilleret *et al*[53]	1991	14	Left colon resection	Intracolonic bypass	0 AL
Ravo *et al*[34]	1985	29	Left colon or rectal resection	Intracolinic bypass	0 AL
Ravo[29]	1988	Case report	Sigmoid resection	Intracolonic bypass	0 AL
Keane *et al*[37]	1988	6	Sigmoid or rectal resection	Intracolinic bypass	0 AL
Rosati *et al*[36]	1992	29	Left colon or rectal resection	Intracolinic bypass	2 AL (7%)
Egozi *et al*[38]	1993	Case report	Sigmoid	Intracolinic bypass	Colon necrosis at site of tube
Yoon *et al*[41]	1994	10	LAR	Condom	0 AL
Sterk *et al*[19]	2001	50	LAR	Transanal tube	3 Grade A AL (6%) 2 Grade C AL (4%)
Amin[1] *et al*[20]	2003	76	LAR	41 transanal stent 35 loop stoma	Stent: 3 Grade C AL (7%) Stoma: 1 Grade A AL (3%) 1 Grade C AL (3%)
Bülow[2] *et al*[21]	2006	194	LAR	98 Transanal stent 96 controls	Stent: 17 AL (17%) Control: 8 AL (8%)
Ye[3] *et al*[47]	2008	83	LAR	44 VIB 39 Loop ileostomy	VIB: 2 Grade A AL (5%) Stoma: 2 Grade A AL (5%)
Kolkert *et al*[48]	2010	15	Sigmoid or rectal resection	C-seal	0 AL

[1]Randomized trial; [2]Randomized trial, with/without stent and with/without ostomy; [3]Patient could choose between VIB and LI. AL:Anastomotic leakage, according to the ISREC classification[12]; LAR: Low anterior resection; VIB: Valtrac-secured intracolonic bypass; LI: Loop ileostomy; ISREC: International Study Group of Rectal Cancer.

Figure 1 Transanal stent.

In 1978, Balz *et al*[18] reviewed a series of 392 patients undergoing anterior resection with placement of an indwelling rectal tube. Anastomotic complications occurred in 3.8%. In addition to decompression, the rectal tube facilitated intraluminal antibiotic irrigation of the anastomosis.

Transanal rubber drain: In 2001, Sterk *et al*[19] used a transanal rubber drain to protect the anastomosis after low anterior resection in 50 patients. The maximal distance between the anastomosis and the anal skin was 7 cm. The transanal rubber drain had openings on the side, a length of 40 cm, and a diameter of 12-15 mm. The tip of the drain was positioned about 10 cm proximal to the anastomosis; the other end was fixed to the perineal skin. Two patients (4%) developed a grade C AL and three patients (6%) a grade A AL. The authors concluded that the transanal drain was at least equivalent to a conventional colostomy to reduce symptomatic AL.

Human trials

Transanal stent: The transanal stent (TAS) is a radio-opaque soft silicone tube, 4 cm in length with funnel-shaped flanges. It is inserted into the anal canal at the end of the procedure, and is left *in situ* for 5-7 d (Figure 1). Amin *et al*[20] performed a randomised trial with the TAS in LAR for rectal cancer. Forty-two of 118 patients were not randomised because of high dose pre-operative radiotherapy, concern about the anastomosis, or obstructing tumours. Seventy-six patients were randomised to TAS or a proximal defunctioning loop stoma (LS). No significant difference in AL rate was demonstrated between the two groups (TAS: three AL's, all grade C; LS: two AL's grade A and C). Patients with a TAS had fewer general infectious complications (17% *vs* 35%) and a shorter hospital stay (13 *vs* 23 d; $P < 0.001$). This study is one of the few trials that actually tested an intracolonic device. Unfortunately, the randomisation strategy is not very clear and no control group with patients without an LS or TAS were described.

In 2006, Bülow *et al*[21] performed a prospective randomised trial to evaluate TAS in patients undergoing anterior resection for a mobile rectal tumour. The use of a protective ileostomy was left to the discretion of the operating surgeon. After completion of the opera-

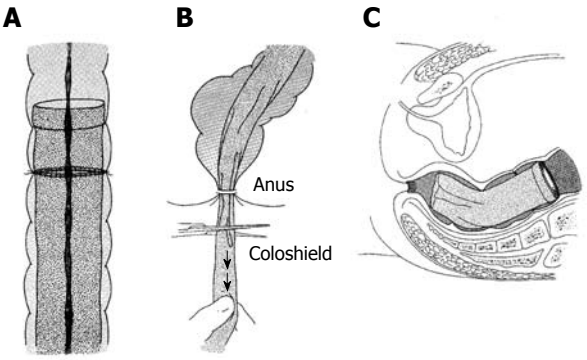

Figure 2 The Coloshield. A: The coloshield is sutured to the submucosa of the bowel proximal of the anastomosis; B and C: Slight traction is placed on the coloshield and it is cut so that it lies in the rectal ampulla.

tion, patients were randomised to the TAS group or the control group. After inclusion of 194 patients (of the planned 448 patients) an interim analysis was performed because of the occurrence of several leaks over a short time. Clinically significant leakage was diagnosed in 25 patients (13%), of whom seven were treated with drainage only and 18 with relaparotomy. AL occurred more frequently in the TAS groups (17%) than in the control group (8%). Although the difference in leak rate appears to be clinically relevant, the difference was not statistically significant because of the small sample size ($P = 0.09$). The study was stopped prematurely for ethical reasons because of this trend. Although this study was unable to demonstrate a statistical difference in AL between the groups, it seems unlikely that inserting the TAS reduces the incidence of AL (Figure 1).

INTRALUMINAL DEVICES

Intracolonic devices do not aim at preventing anastomotic dehiscence. However, they may prevent the faecal load from contacting the anastomotic site, thereby preventing leakage of faeces into the peritoneal cavity when the walls of the anastomosis have become dehiscent. When the faecal stream is bypassed from contacting the bowel mucosa, a gap in the anastomosis will not lead to extravasation of intraluminal content. Shielding the anastomosis from contact with faeces might also reduce the incidence of AL[22].

Animal studies

Coloshield: In the 1980's, Ravo and Ger developed an intraluminal colonic tube to prevent anastomotic leakage. The application procedure is illustrated in Figure 2. After the bowel resection, the proximal loop is inverted for 4-6 centimetres. The proximal end of the tube, reinforced with a cloth strip, is fixed to the proximal bowel loop using polyglycolic acid sutures. The inverted intestinal portion is overturned to its normal anatomic position and the posterior half of the anastomosis is performed. Then, a rectal probe is introduced through the anus and is connected to the tube by a built-in connector. The probe is drawn outside the intestinal lumen through the distal bowel and the anastomosis is completed by suturing the anterior part. The tube is then cut at the level of the anal orifice after a light traction, spontaneously returning inside. In cases where the mid-or lower rectum is resected, the tube is left protruding from the anus and an incontinence bag is attached to the perineum[23]. Studies on dogs were performed using different tubes varying in width and length, material (latex, silicone, rubber), and suture technique. The colon tube placement was found to be a safe, uncomplicated procedure and none of the dogs (three studies, 14 dogs per study) developed AL (evaluated by laparotomy and barium studies). All tubes were expelled naturally together with the faecal stream[24-26]. Even when an intentionally incomplete anastomosis was made after inserting the tube, no AL occurred. In 1985, the bypass tube was successfully tested on five dogs using a (circular) stapler[27].

Silicone prosthesis: In 1992, Serra *et al*[28] studied the efficacy of intracolonic silicone prosthesis in 42 dogs. The use of the prosthesis is similar to the technique described by Ravo and Ger[23,26,29]. The primary objective of the study was to evaluate the efficacy of the intracolonic silicone prosthesis in protecting the anastomosis. Three groups of 14 dogs each (colonic occlusion, diverticulitis, and control) were randomized to undergo resection and anastomosis with or without the silicone prosthesis. A significant difference in mortality was found: six dogs without prosthesis developed anastomotic failure, of which three died. No deaths or AL occurred in the prosthesis groups.

Soft latex tube: Intraluminal colonic tubes were studied by Ross[30] in a rat model. The rats were divided in four groups; all underwent colon diversion with creation of an incomplete anastomosis. The first group consisted of rats treated with an intracolonic tube made from rat duodenum. In the second group, an intracolonic soft latex tube was introduced. The third group had a tube placed outside the colon lumen, and the last group was a control group with an incomplete anastomosis. The tubes were attached 1.5 cm above the incomplete anastomosis. The tubes remained inside the rectum, barely reaching the dentate line and were removed after five or six days. Rats treated with latex and rat duodenum tubes showed a better survival compared to controls (52% and 71%, respectively *vs* 25% in controls). Rats treated with rat duodenum showed a significant better survival compared to the control group ($P < 0.02$). A mortality rate of 100% was found in rats with a tube placed outside the lumen of the colon. The results suggest that only intraluminal tubes have a survival advantage compared to controls. This finding may be explained by the fact that tubes will prevent faecal contamination of the anastomotic site and allow time for secondary healing of the anastomosis.

Figure 3 Polyflex stents with a proximal flare.

Polyflex self-expandable covered plastic stent: As a result of achievements in biomedical technology, in 2008 Tsereteli *et al*[31] performed a randomized controlled trial in 16 pigs comparing the incidence of AL after open rectosigmoid resection with or without a 21 mm Polyflex self-expandable covered plastic stent (Figure 3). The stent was placed over a guidewire with use of a flexible colonoscope and deployed under fluorescence control. A 2-cm anastomotic gap was created. After 6-9 d, stents were spontaneous expelled. At autopsy, none of the animals in the study group (n = 8) showed leak-related complications, although two pigs developed an unrelated postoperative complication (evisceration and bladder necrosis) and died. Five out of eight control animals (63%) showed intra-abdominal infection around the anastomosis at autopsy, with four abscesses and one fistula. This demonstrated a significant beneficial effect of the stent group *vs* controls (P = 0.002). The authors stated that the stent could be a breakthrough solution for the complicated colorectal anastomosis, avoiding the necessity of a stoma during the healing process. A potential new indication for this stent was also to seal an acute anastomotic leak, which is supported by one case report describing the successful use of a coated stent in healing a 1-cm fistula from a rectosigmoid anastomosis two weeks after surgery[32]

Human studies

Coloshield: The development of the intraluminal tube led to the final version of the Coloshield: a soft, pliable tube like a surgical glove. This intraluminal protective device, developed by Ravo, was first used in humans in 1984[25,33]. Indications for use include perforated diverticulitis, colonic obstruction, volvulus, carcinoma, and fistula. Several non-randomized studies were performed in patients (ranging from n = 6 to n = 98) undergoing colon surgery with the Coloshield. The reported anastomosis-related complication rates varied between 0% and 8.7%[23,25,34-37]. The Coloshield related complications included two anastomotic dehiscences (from a group of 98 patients) following low anterior resection, both attributed to technical errors[23]. Egozi *et al*[38] described a case with a complicated course after insertion of a rigid intracolonic bypass. On the 8th postoperative day, the Coloshield was found to have eroded through the colon. Castrini *et al*[33] tested an intracolonic latex bypass in 19 patients undergoing left colon or rectal resection. None of these patients developed anastomotic complications. Regrettably, no detailed information concerning procedures, patients, and complications was reported.

The last article concerning the Coloshield was published by Ravo in 1991. Ravo described a method of inserting the Coloshield in the proximal colon after completion of the anastomosis by performing a longitudinal colostomy on the antimesenteric border of the afferent loop, proximal to the anastomosis[39]. Ravo and Ger pioneered the use of intracolonic stents, testing different materials (silicone, rubber, and latex) before developing and, finally, filing the patent of the latex Coloshield[40]. They concluded that the one-stage intracolonic bypass procedure is a viable alternative to the two- or three-stage procedure because it reduces the length of hospital stay and the length of disability. Despite its promise, the Coloshield has not been widely accepted. Ravo still uses the Coloshield (personal communication).

Condom: In 1994, Yoon *et al*[41] used a condom instead of a Coloshield to protect the colo-anal anastomosis. Ten patients with rectal carcinoma undergoing LAR received this condom. The ring of the sterilised condom was sutured to the mucosal and submucosal layer of the proximal colon before completing the anastomosis. The condom was brought to the exterior and transected with scissors. The device is expelled naturally from the anus between the 10th and 14th postoperative day. No anastomotic dehiscence, leakage, or colonic necrosis occurred. In 1995, Ruiz *et al*[42] described the same method using a condom (termed a skinless skin) as a protective device in a colonic anastomosis. When using a stapler, the distal end of the condom is attached to the anvil of the EEA stapler with two stay sutures, permitting it to be pulled through the anastomosis. Ruiz *et al* hypothesized that a latex condom is a cheap and safe device that decreases the risk of dehiscence and permits the performance of a large number of primary anastomoses. Unfortunately, for both studies, no detailed information on the procedures and patients are available.

BIODEGRADABLE DEVICES

Animal studies

In 1993, Winkeltau tested the protective effect of biodegradable bipolymer intraluminal stents in 90 rats under the adverse condition of induced general peritonitis (verified by inspection, microbiology, and histology)[43]. Peritonitis was induced using the cecal ligation and puncture model[44]. Stents of various shapes and biodegradable materials were compared to controls with no stents, in rats undergoing jejuno-jejunostomy. The survival rate in the control group was 25% and rats receiving a tube had a significantly better survival, varying between

Figure 4 Murphy's button. John Benjamin Murphy developed his device in 1892 as a quick and safe method of intestinal anastomosis. The steel Murphy button had two rounded heads mounted on hollow shafts. After the intestinal ends were tied on the shafts, the heads were screwed together to compress the tissue.

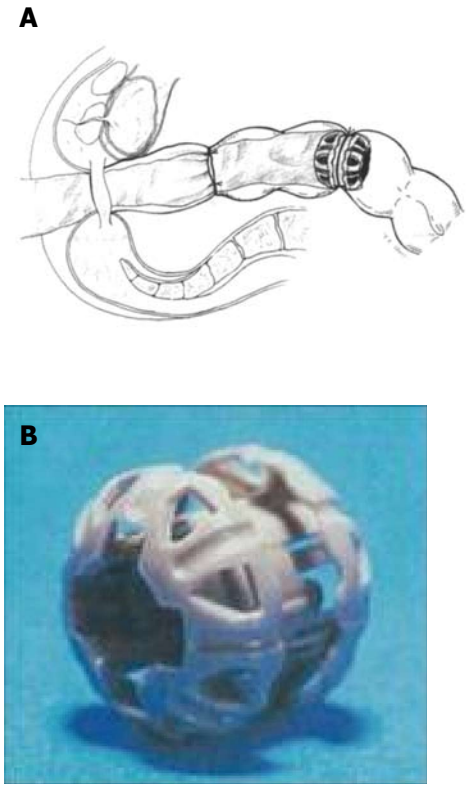

Figure 5 Valtrac-secured intracolonic bypass device. A: Rough colorectal anastomosis with large gaps between sutures protected by the intracolonic bypass; B: Biofragmentable anastomosis ring.

65% and 90%. The best results were obtained in rats with a funnel shaped BCL-004 tube, mainly composed of polyhydroxybutyric acid (PHB). The use of degradable materials is not restricted to the distal parts of the gastrointestinal tract, since it does not carry the risk of causing an obstruction.

Valtrac secured intracolonic bypass (VIB): Chen[45] introduced the VIB, which consists of a soft vinyl tube attached to a biofragmentable anastomosis ring (BAR). The BAR was introduced by Hardy *et al*[46] in 1985 (inspired by Murphy's button). The BAR realises a sutureless intestinal anastomosis composed of two identical segments. The two components interdigitate and can be approximated to a semi-closed position with a 6 mm gap between the two edges of the ring (Figures 4 and 5).

Chen attached the BAR to the lumen of a pig's colon, 5-10 cm proximal from the anastomotic site, by putting a simple suture encircling the colon at the site of the BAR gap. The tube attached to the BAR passed through the anastomosis to the anus, thereby preventing contact between the anastomotic site and the faecal stream. Eighteen pigs underwent colonic resection with the deliberate creation of an incomplete anastomosis. Six pigs received the bypass, six received the bypass under the condition of a colonic outlet obstruction (created by tying a purse string suture at the level of the anus) and six pigs were controls. All pigs with the bypass had no anastomotic leakage (checked by a barium enema) and survived. Temporary anorexia and abdominal distension were noted in pigs with a colonic outlet obstruction. Four of six controls developed anastomotic leakage, of which three died.

Human studies

Valtrac secured intracolonic bypass: In 2002, the VIB was tested on 83 patients undergoing LAR for rectal cancer[47]. After inclusion, the patient decided whether he/she wanted to be treated with the VIB or with a loop ileostomy (LI) to protect the anastomosis. The VIB was attached to the colon 5-7 cm proximal of the anastomosis by the same method Chen *et al* used in their experimental study. The fragmentation and excretion of the BAR occurred 12-22 d postoperatively. Fifty-three percent of patients chose the VIB and 47% chose the LI as treatment. Four subclinical anastomotic dehiscences were diagnosed, two in each group. Total hospital stay and costs were significant lower in the VIB group (P = 0.001); no readmission for a take down of the stoma was indicated. In two patients, the BAR detached en-bloc, which led to a difficult expulsion. In these cases the BAR was manually crushed and excreted through the anus. The authors concluded that the VIB is a safe and effective diverting technique to protect an elective low colorectal anastomosis; it avoids stoma-related complications and lowers the cost. This study can be criticized because the lack of randomization and high probability of introduction of bias.

C-seal: A recent development from the Groningen group is the C-seal: a thin walled tube like a soft sheet or condom, with a diameter of 4 cm, a length of 25 cm and a wall thickness of 70 μm (Figure 6). The C-seal is a tubular device composed of a biodegradable synthetic material. Two flaps with adhesive tape are located at one end of the tube. These flaps are used to attach the C-seal to the stapler cap, to facilitate an easy pull-through of the C-seal after the anastomosis is made (http://www.jove.com/index/details.stp?ID=2223). The C-seal remains

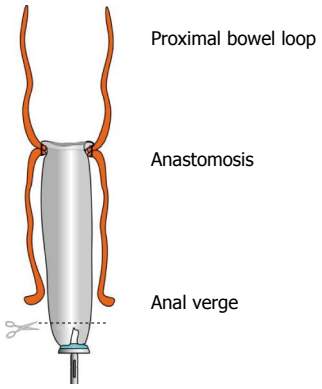

Figure 6 C-seal: A biodegradable drain protecting the anastomosis.

in place for about 10 d, according to the engineered composition of the biodegradable material. Thereafter, it looses strength, degrades, and is secreted from the body together with the gastrointestinal natural contents. In 2007, a pilot study was performed testing the C-seal in 15 patients diagnosed with colorectal carcinoma undergoing LAR with stapled anastomoses[48]. No (sub) clinical AL was diagnosed in these 15 patients. Currently, the C-seal is being tested in a second phase study of 35 patients undergoing (colo-)rectal resection with stapled colorectal anastomosis.

CONCLUSION

The relative high incidence of anastomotic leakage after colorectal surgery, with its major consequences for morbidity and mortality, remain of great concern. Some authors concentrate on early detection of anastomotic dehiscence to reduce the consequences of AL[49-52]. The ideal situation, however, would be prevention of anastomotic leakage. Many devices have been developed to prevent AL by protecting the anastomotic site. We categorised these devices as transanal decompression, intraluminal, and biodegradable protective devices. A number of studies concerning intraluminal tubes demonstrate low leakage rates[18,33-35,48]. Despite these positive results, the use of protective devices has not been widely implemented. Clinicians are probably reluctant to use these devices in clinical practice for a number of reasons.

First, the use of intraluminal devices has only a small basis of evidence in the literature. Most papers are either animal studies or small, non-randomized human studies, often without a control group. Furthermore, most studies are heterogeneous and use different devices[33,37,41,53]. Only two randomized, controlled studies are published, both on decompression devices. Amin *et al*[20] compared a defunctioning stoma with the transanal stent. This study does not show a benefit of the transanal stent. The study suffers from unclear eligibility criteria and a non-transparent randomisation process: one-third of the registered patients were not randomised. With 76 evaluable patients, the study is not sufficiently powered to detect significant differences in leakage rate between the groups. A similar study by Bülow[21] was prematurely stopped due to a high overall leakage rate, with a trend for a higher leakage rate in the TAS group.

Contrary to the transanal stent, a number of papers suggest that the Coloshield may help to reduce AL, though this beneficial effect has only been demonstrated in small studies with no control group[33-35,39,41,53]. Unfortunately, no proper, randomised trial comparing the Coloshield to the standard of care has been performed until now.

Another aspect of the Coloshield is that it is considered time-consuming and tedious to apply, making it less attractive than the standard procedure. Tsereteli hypothesized that the Coloshield never found wide acceptance because of its technical difficulties and the requirement of a laparotomy for placement[31]. Finally, medical devices are often only successfully introduced by companies who can organize an optimal marketing campaign and a widespread network of representatives. According to Ravo, the Coloshield was never widely accepted because it lacked these factors. Nevertheless, Ravo still uses the Coloshield in daily practice (personal communication).

We conclude that there is currently no high-level evidence demonstrating a benefit of intraluminal devices to reduce AL. Based on the literature, we think that the intraluminal device holds clinical promise to reduce or prevent early leakage of colo-rectal anastomoses and concomitant sequelae (Table 1). Although a number of very innovative approaches have been reported, not all devices have been appropriately studied in a randomized, controlled fashion with sufficient power to rule out chance or bias.

REFERENCES

1 **Jung SH**, Yu CS, Choi PW, Kim DD, Park IJ, Kim HC, Kim JC. Risk factors and oncologic impact of anastomotic leakage after rectal cancer surgery. *Dis Colon Rectum* 2008; **51**: 902-908
2 **Peeters KC**, Tollenaar RA, Marijnen CA, Klein Kranenbarg E, Steup WH, Wiggers T, Rutten HJ, van de Velde CJ. Risk factors for anastomotic failure after total mesorectal excision of rectal cancer. *Br J Surg* 2005; **92**: 211-216
3 **Matthiessen P**. Risk factors for anastomotic leakage after anterior resection of the rectum. *Colorectal Dis* 2006; **8**: 366
4 **Dehni N**, Schlegel RD, Cunningham C, Guiguet M, Tiret E, Parc R. Influence of a defunctioning stoma on leakage rates after low colorectal anastomosis and colonic J pouch-anal anastomosis. *Br J Surg* 1998; **85**: 1114-1117
5 **Laxamana A**, Solomon MJ, Cohen Z, Feinberg SM, Stern HS, McLeod RS. Long-term results of anterior resection using the double-stapling technique. *Dis Colon Rectum* 1995; **38**: 1246-1250
6 **Marusch F**, Koch A, Schmidt U, Geibetaler S, Dralle H, Saeger HD, Wolff S, Nestler G, Pross M, Gastinger I, Lippert H. Value of a protective stoma in low anterior resections for rectal cancer. *Dis Colon Rectum* 2002; **45**: 1164-1171
7 **Hüser N**, Michalski CW, Erkan M, Schuster T, Rosenberg R, Kleeff J, Friess H. Systematic review and meta-analysis of the role of defunctioning stoma in low rectal cancer surgery. *Ann Surg* 2008; **248**: 52-60
8 **Mealy K**, Burke P, Hyland J. Anterior resection without a defunctioning colostomy: questions of safety. *Br J Surg* 1992;

79: 305-307
9 **Bailey CM**, Wheeler JM, Birks M, Farouk R. The incidence and causes of permanent stoma after anterior resection. *Colorectal Dis* 2003; **5**: 331-334
10 **Peel AL**, Taylor EW. Proposed definitions for the audit of postoperative infection: a discussion paper. Surgical Infection Study Group. *Ann R Coll Surg Engl* 1991; **73**: 385-388
11 **Bruce J**, Krukowski ZH, Al-Khairy G, Russell EM, Park KG. Systematic review of the definition and measurement of anastomotic leak after gastrointestinal surgery. *Br J Surg* 2001; **88**: 1157-1168
12 **Rahbari NN**, Weitz J, Hohenberger W, Heald RJ, Moran B, Ulrich A, Holm T, Wong WD, Tiret E, Moriya Y, Laurberg S, den Dulk M, van de Velde C, Büchler MW. Definition and grading of anastomotic leakage following anterior resection of the rectum: a proposal by the International Study Group of Rectal Cancer. *Surgery* 2010; **147**: 339-351
13 **Fraser I**. An historical perspective on mechanical aids in intestinal anastomosis. *Surg Gynecol Obstet* 1982; **155**: 566-574
14 **Gurjar SV**, Forshaw MJ, Ahktar N, Stewart M, Parker MC. Indwelling trans-anastomotic rectal tubes in colorectal surgery: a survey of usage in UK and Ireland. *Colorectal Dis* 2007; **9**: 47-51
15 **Goldman G**, Aladgem D, Kahn PJ, Wiznitzer T. Intrarectal bypass graft in low anterior resection and sigmoid obstruction--an experimental study. *Eur Surg Res* 1988; **20**: 238-242
16 **Stewart WR**, Samson RB. Rectal tube decompression of left-colon anastomosis. *Dis Colon Rectum* 1968; **11**: 452-456
17 **Rack RJ**. Advantages of an indwelling rectal tube in anterior resection and anastomosis for lesions involving the terminal portion of the colon. *Dis Colon Rectum* 1966; **9**: 42-48
18 **Balz J**, Samson RB, Stewart WR. Rectal-tube decompression in left colectomy. *Dis Colon Rectum* 1978; **21**: 94-97
19 **Sterk P**, Schubert F, Günter S, Klein P. [Anastomotic protection with a transanal tube after rectum resection and total mesorectal excision]. *Zentralbl Chir* 2001; **126**: 601-604
20 **Amin AI**, Ramalingam T, Sexton R, Heald RJ, Leppington-Clarke A, Moran BJ. Comparison of transanal stent with defunctioning stoma in low anterior resection for rectal cancer. *Br J Surg* 2003; **90**: 581-582
21 **Bülow S**, Bulut O, Christensen IJ, Harling H. Transanal stent in anterior resection does not prevent anastomotic leakage. *Colorectal Dis* 2006; **8**: 494-496
22 **Ravo B**, Metwally N, Castera P, Polansky PJ, Ger R. The importance of intraluminal anastomotic fecal contact and peritonitis in colonic anastomotic leakages. An experimental study. *Dis Colon Rectum* 1988; **31**: 868-871
23 **Ravo B**. The intracolonic bypass procedure. *Int J Colorectal Dis* 1987; **2**: 38-42
24 **Ger R**, Ravo B. Prevention and treatment of intestinal dehiscence by an intraluminal bypass graft. *Br J Surg* 1984; **71**: 726-729
25 **Ravo B**, Ger R. A preliminary report on the intracolonic bypass as an alternative to a temporary colostomy. *Surg Gynecol Obstet* 1984; **159**: 541-545
26 **Ravo B**, Ger R. Intracolonic bypass by an intraluminal tube. An experimental study. *Dis Colon Rectum* 1984; **27**: 360-365
27 **Ravo B**, Ger R. Anastomosis of intracolonic bypass tube by the use of EEA stapler. An experimental study. *Ital J Surg Sci* 1986; **16**: 179-183
28 **Serra J**, Capella G, Esquius J, Montañes R, Rius X. Experimental study of the efficacy of the endoluminal prosthesis in colonic anastomoses. *Int J Colorectal Dis* 1992; **7**: 21-25
29 **Ravo B**. Colorectal anastomotic healing and intracolonic bypass procedure. *Surg Clin North Am* 1988; **68**: 1267-1294
30 **Ross H**. The effect of an intraluminal tube used as an internal drain on the healing of the rat colon. *Dis Colon Rectum* 1987; **30**: 591-594
31 **Tsereteli Z**, Sporn E, Geiger TM, Cleveland D, Frazier S, Rawlings A, Bachman SL, Miedema BW, Thaler K. Placement of a covered polyester stent prevents complications from a colorectal anastomotic leak and supports healing: randomized controlled trial in a large animal model. *Surgery* 2008; **144**: 786-792
32 **Scileppi T**, Li JJ, Iswara K, Tenner S. The use of a Polyflex coated esophageal stent to assist in the closure of a colonic anastomotic leak. *Gastrointest Endosc* 2005; **62**: 643-645
33 **Castrini G**, Ger R, Pappalardo G, Ravo B, Trentino P, Pisapia M. Intracolonic by-pass: a new technique to prevent anastomotic complications in colon and rectal surgery. *Ital J Surg Sci* 1984; **14**: 189-193
34 **Ravo B**, Ger R. Temporary colostomy--an outmoded procedure? A report on the intracolonic bypass. *Dis Colon Rectum* 1985; **28**: 904-907
35 **Ravo B**, Mishrick A, Addei K, Castrini G, Pappalardo G, Gross E, Sackier JM, Wood CB, Ger R. The treatment of perforated diverticulitis by one-stage intracolonic bypass procedure. *Surgery* 1987; **102**: 771-776
36 **Rosati C**, Smith L, Deitel M, Burul CJ, Baida M, Borowy ZJ, Bryden P. Primary colorectal anastomosis with the intracolonic bypass tube. *Surgery* 1992; **112**: 618-622; discussion 622-623
37 **Keane PF**, Ohri SK, Wood CB, Sackier JM. Management of the obstructed left colon by the one-stage intracolonic bypass procedure. *Dis Colon Rectum* 1988; **31**: 948-951
38 **Egozi L**, Sorrento JJ, Golub R, Schultz EH. Complication of the intracolonic bypass. Report of a case. *Dis Colon Rectum* 1993; **36**: 191-193
39 **Ravo B**, Reggio D, Frattaroli FM. Insertion of the Coloshield through a colotomy after completion of a colonic anastomosis. *Int J Colorectal Dis* 1991; **6**: 46-48
40 **Ravo B**. Intracolonic bypass procedure. *Dis Colon Rectum* 1997; **40**: 628-629
41 **Yoon WH**, Song IS, Chang ES. Intraluminal bypass technique using a condom for protection of coloanal anastomosis. *Dis Colon Rectum* 1994; **37**: 1046-1047
42 **Ruiz PL**, Facciuto EM, Facciuto ME, Rodriguez Otero JC, Pigatto J, Cominelli H. New intraluminal bypass tube for management of acutely obstructed left colon. *Dis Colon Rectum* 1995; **38**: 1108-1109
43 **Winkeltau GJ**, Treutner KH, Kleimann E, Lerch MM, Ger R, Haase G, Schumpelick V. Protection of intestinal anastomoses by biodegradable intraluminal bypass tubes under the condition of general peritonitis: an experimental study on the CLP model in rats. *Dis Colon Rectum* 1993; **36**: 154-160
44 **Wichterman KA**, Baue AE, Chaudry IH. Sepsis and septic shock--a review of laboratory models and a proposal. *J Surg Res* 1980; **29**: 189-201
45 **Chen TC**, Yang MJ, Chen SR, Chang CP, Chi CH. Valtrac-secured intracolonic bypass device: an experimental study. *Dis Colon Rectum* 1997; **40**: 1063-1067
46 **Hardy TG**, Pace WG, Maney JW, Katz AR, Kaganov AL. A biofragmentable ring for sutureless bowel anastomosis. An experimental study. *Dis Colon Rectum* 1985; **28**: 484-490
47 **Ye F**, Wang D, Xu X, Liu F, Lin J. Use of intracolonic bypass secured by a biodegradable anastomotic ring to protect the low rectal anastomosis. *Dis Colon Rectum* 2008; **51**: 109-115
48 **Kolkert JL**, Havenga K, ten Cate Hoedemaker HO, Zuidema J, Ploeg RJ. Protection of stapled colorectal anastomoses with a biodegradable device: the C-Seal feasibility study. *Am J Surg* 2011; **201**: 754-758
49 **Matthiessen P**, Henriksson M, Hallböök O, Grunditz E, Norén B, Arbman G. Increase of serum C-reactive protein is an early indicator of subsequent symptomatic anastomotic leakage after anterior resection. *Colorectal Dis* 2008; **10**: 75-80
50 **Matthiessen P**, Strand I, Jansson K, Törnquist C, Andersson M, Rutegård J, Norgren L. Is early detection of anastomotic leakage possible by intraperitoneal microdialysis and intra-

peritoneal cytokines after anterior resection of the rectum for cancer? *Dis Colon Rectum* 2007; **50**: 1918-1927
51 **Komen N**, de Bruin RW, Kleinrensink GJ, Jeekel J, Lange JF. Anastomotic leakage, the search for a reliable biomarker. A review of the literature. *Colorectal Dis* 2008; **10**: 109-115; discussion 115-117
52 **den Dulk M**, Noter SL, Hendriks ER, Brouwers MA, van der Vlies CH, Oostenbroek RJ, Menon AG, Steup WH, van de Velde CJ. Improved diagnosis and treatment of anastomotic leakage after colorectal surgery. *Eur J Surg Oncol* 2009; **35**: 420-426
53 **Cuilleret J**, Burgard G, Berger JL, Bou B. [Endoluminal protection of colorectal anastomosis by Coloshield. Apropos of 14 cases]. *J Chir* (*Paris*) 1991; **128**: 351-355

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ORIGINAL ARTICLE

Fascin promotes the motility and invasiveness of pancreatic cancer cells

Yan-Feng Xu, Shuang-Ni Yu, Zhao-Hui Lu, Jian-Ping Liu, Jie Chen

Yan-Feng Xu, Shuang-Ni Yu, Zhao-Hui Lu, Jian-Ping Liu, Jie Chen, Department of Pathology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tsinghua University, Beijing 100730, China

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Correspondence to: Dr. Jie Chen, Department of Pathology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tsinghua University, Beijing 100730, China. xhblk@163.com
Telephone: +86-10-65295490 Fax: +86-10-65295490

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Abstract

AIM: To explore the role of actin-bundling protein, fascin during the progression of pancreatic cancer.

METHODS: The plasmid expressing human fascin-1 was stably transfected into the pancreatic cancer cell line MIA PaCa-2. The proliferation, cell cycle, motility, scattering, invasiveness and organization of the actin filament system in fascin-transfected MIA PaCa-2 cells and control non-transfected cells were determined.

RESULTS: Heterogeneous overexpression of fascin markedly enhanced the motility, scattering, and invasiveness of MIA PaCa-2 cells. However, overexpression of fascin had minimal effect on MIA PaCa-2 cell proliferation and cell cycle. In addition, cell morphology and organization of the actin filament system were distinctly altered in fascin overexpressed cells. When transplanted into BALB/c-nu mice, fascin-transfected pancreatic cancer cells developed solid tumors at a slightly slower rate, but these tumors displayed more aggressive behavior in comparison with control tumors.

CONCLUSION: Fascin promotes pancreatic cancer cell migration, invasion and scattering, thus contributes to the aggressive behavior of pancreatic cancer cells.

Key words: Fascin; Invasiveness; Motility; Pancreatic cancer

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INTRODUCTION

Pancreatic cancer is characterized by aggressiveness and early metastasis, and the survival rate for this cancer is among the lowest of all cancer types. In an effort to elucidate additional targets for the detection and therapy for this type of cancer, our lab completed a proteomic analysis of primary pancreatic cancer and normal pancreas samples[1]. We identified 70 proteins that were expressed at least 2-fold higher in pancreatic cancers when compared with normal pancreas samples. Of these proteins,

18 were involved in cytoskeleton regulation, and fascin was one of the identified proteins that had the greatest change between pancreatic cancer and normal pancreas samples. Because cell motility is based on rearrangement of the actin cytoskeleton and this process of rearrangement is governed by multiple actin-binding proteins, we postulated that these proteins may play some role in the invasion and metastasis of pancreatic cancer. Several other studies have previously shown that the actin-bundling protein, fascin, which is specifically expressed in pancreatic cancer when compared with normal pancreas, is closely associated with the status of pancreatic cancer cell differentiation and plays an important role in pancreatic cancer progression[2-5].

Fascin was identified in the 1970s to be a 55-kD globular protein that cross-links F-actin into well-ordered and tightly packed parallel bundles that are concentrated in cell protrusions during cell migration. Fascin is highly expressed in specialized cells that are rich in filopodia, such as neurons, glial cells, mature dendritic cells and actively migrating cells, such as the endothelial cells of microvessels[6,7]. Fascin expression is often absent in normal epithelial cells, such as the epithelia of the bile duct[8], urinary bladder[9], breast[10], colon[11], ovary[12], pancreas[1] and stomach[13]. Fascin expression is upregulated in several human neoplasms, such as breast[10], lung[14], kidney[15], ovary[12], prostate and pancreatic cancers[3,5,16]. Fascin overexpression is often correlated with an invasive tumor phenotype, poor prognosis and decreased disease-free survival.

The role of fascin in the malignant behavior of pancreatic cancer remains unknown. To determine the functional consequences of fascin overexpression in pancreatic cancer cells, we stably transfected a human pancreatic cancer cell line, MIA PaCa-2, with a plasmid containing full-length human fascin cDNA. The proliferation, cell cycle, motility, scattering, invasiveness and organization of the actin filament system were evaluated in fascin-transfected MIA PaCa-2 cells and in non-transfected control cells.

MATERIALS AND METHODS

Cell culture

The human pancreatic cancer cell lines, BxPC-3, MIA PaCa-2 and AsPC-1 were obtained from American Type Culture Collection (Rockville, MD, United States), and the PC-1, PC-4 and PC-7 cell lines were established and maintained in our laboratory. The BxPC-3, AsPC-1, PC-1, PC-4 and PC-7 cell lines were cultured in RPMI 1640 (GIBCO, Paisley, United Kingdom) with 10% fetal bovine serum (FBS) (HyClone Laboratories, United States) and penicillin-streptomycin (100 IU/mL-0.1 mg/mL). The MIA PaCa-2 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Paisley, United Kingdom) supplemented with 10% FBS and penicillin-streptomycin (100 IU/mL-0.1 mg/mL). All cells were maintained in a humidified 5% CO_2 atmosphere at 37 ℃.

Antibodies

The fascin antibody (M3567) was purchased from DAKO (Glostrup, Denmark); the β-actin antibody was purchased from Sigma (MO, United States); the EnVasion™ Detection Kit was purchased from DAKO (Glostrup, Denmark); and the FITC-conjugated secondary antibody was purchased from Boster (WuHan, China).

Transfection

A pcDNA3 vector containing the full-length human fascin cDNA (pcDNA3-Fascin) was kindly provided by Dr. Josephine C. Adams (Lener Research Institute, Cleveland, Ohio, United States). The insert was cut out with the EcoR I restriction enzyme to acquire the pcDNA3 control vector (pcDNA3-Vector). The sequence was verified by DNA sequencing. MIA PaCa-2 cells were transfected with either pcDNA3-Fascin or pcDNA3-Vector. Approximately 5×10^4 MIA PaCa-2 cells per well were seeded in a 6-well culture plate and were subsequently transfected with 5 μg of plasmid using 10 μL of Lipofectamine 2000 (GIBCO, United States) in 250 μL of Opti-MEM (GIBCO, United States). After 48 h, G418 (GIBCO, United States) was added to the cells for selection at a concentration of 800 μg/mL. After 10 to 14 d, antibiotic-resistant colonies were picked, pooled and maintained in DMEM containing 10% FBS and 400 μg/mL G418.

Western blotting analysis

Cells were rinsed twice with D-Hanks and solubilized with lysis buffer [50 mmol/L Tris (pH 8.0), 1% Nonidet p-40, 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate, 0.5% deoxysodium cholate, 1 × cocktail (Roche, Mannheim, Germany)] for 30 min on ice. The total extract was cleaned by centrifugation at 12 000 r/min for 30 min at 4 ℃, and the supernatant was collected. The protein concentration was determined with the Bradford assay (BioRad, CA, United States). A total of 40 μg of total cell extract was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were transferred onto an Immobilon-PVDF membrane (Millipore, Bedford, MA, United States) and were blocked and incubated with the primary antibody overnight at 4 ℃. The Envasion™ Detection Kit with DAB liquid substrate (DAKO, Glostrup, Denmark) was used for protein detection.

Immunocytochemistry and Immunofluorescence

Cells were cultured on sterile coverslips and were incubated for 24 h in a humidified 5% CO_2 atmosphere at 37 ℃. The coverslips containing the cells were then fixed with 4% paraformaldehyde in phosphate buffered solution (PBS) for 10 min, washed with PBS, permeabilized in 0.2% Triton X-100 in PBS for 5 min, washed and then blocked with normal goat-serum for 30 min at room temperature. Cells were then incubated with an appropriate primary antibody for 1 h at 37 ℃, and were rinsed 3 times with PBS. For protein detection by immunocytochemistry, an Envasion™ Chem™ Detection Kit

(DAKO, Glostrup, Denmark) was used. The reaction color was developed by incubating sections with DAB liquid substrate. The slides were then washed with water and counterstained with hematoxylin. The slides were then dehydrated and mounted with mounting media. For immunofluorescence, a FITC-conjugated goat anti-mouse IgG secondary antibody was used. After washing, the slides were mounted with glycerol and imaged with an immunofluorescence microscope (Olympus BX51).

Proliferation assay

For the proliferation assay, 1×10^4 MIA PaCa-2 cells per well were seeded in a 24-well culture plate in DMEM supplemented with 10% FBS. Every 24 h, cells from 3 independent wells were collected by trypsinization and counted using a hemocytometer.

Wound healing/cell migration assay

Cell migration was evaluated by the wound healing assay[17]. Fascin-transfected MIA PaCa-2 cells and non-transfected control cells were plated separately into 6-well culture plates and cultured to 70%-80% confluence in DMEM containing 10% FBS. After a 24-h serum starvation period, the monolayer of cells was wounded by manual scratching with a sterile plastic 200 μL micropipette tip, washed with PBS 5 times to remove cell debris, photographed with an inverted tissue culture microscope (Leica LEITZDM IL) and then placed in complete medium in a humidified 5% CO_2 atmosphere at 37 ℃. After 20 h of incubation, the wells were re-evaluated under the microscope, and the wounded area was re-imaged for comparison.

Aggregation assay

The ability of the cells to aggregate was tested by hanging drop suspension cultures[18]. Cells were trypsinized with 0.25% trypsin in the presence of 0.01% ethylene diamine tetraacetic acid, washed twice in PBS, and resuspended at 2.5×10^5 cells/mL in DMEM containing 10% FBS. Drops of medium (20 μL in each drop, containing 5000 cells) were pipetted onto the inner surface of a Petri dish lid. The lid was then placed on the Petri dish, and the drops with the cells suspended were left hanging from the lid. To compensate for evaporation, 8 mL of serum-free culture medium was added to the bottom of the Petri dish. After incubation at 37 ℃ for 12 h, the lid of the Petri dish was inverted and photographed under an inverted tissue culture microscope.

Invasion assay

For the invasion assay, the BioCoat Matrigel Invasion Chamber (Becton Dickinson Bioscience, United States) was used according to the manufacturer's instructions. Briefly, 2.5×10^4 MIA PaCa-2 cells suspended in 500 μL serum-free medium were seeded onto Matrigel-coated filters, and 750 μL of DMEM containing 10% FBS was added as a chemoattractant in the lower portion of the wells. After incubation at 37 ℃ with 5% CO_2 for 24 h, the inserts were removed, and the non-invading cells that remained on the upper surface of the filter were scraped off with cotton swabs. Cells on the bottom surface of the membranes were fixed with ethanol and stained with 0.05% crystal violet. The number of cells invading through the Matrigel membrane was counted. Data are presented as the average of triplicate determinants.

Cell cycle analysis

For the cell cycle analysis, a minimum of 1×10^6 cells were harvested and fixed in 70% ethanol at 4 ℃. After 12 h, cells were centrifuged (1000 *g*, 7 min, 4 ℃), resuspended in PBS containing 0.05 mg/mL RNase A (Sigma, United States) and then incubated at room temperature for 30 min. After the cells were washed, they were stained with 10 μg/mL propidium iodide, filtered through a 60 μm mesh, and analyzed by flow cytometry (Elite Epics ESP, Coulter, United States). A total of 10 000 cells were analyzed with MODFIT software.

Xenograft tumor model

All procedures involving mice were approved by the College Committee on Use and Care of Animals at the Peking Union Medical College and conformed to the relevant regulatory standards. Four-week-old male athymic nude (BALB/c-nu) mice (Vitalriver, Beijing) were housed in specific pathogen-free conditions. To generate tumor xenografts, 5×10^6 tumor cells suspended in 0.1 mL of medium were inoculated subcutaneously in the right flank of the mice. Animals were inspected every 3 d. When the tumors from fascin-overexpressing MIA PaCa-2 cells (named MIA PaCa-2 Fascin) or vector control MIA PaCa-2 cells (named MIA PaCa-2-Vector) developed to a visible size, the mice were euthanized, the tumors were collected, cut into 1 mm^3 pieces and then implanted subcutaneously in the right flank of BALB/c-nu mice. A total of 6 mice were used in each group. Animals were inspected and the tumors were measured every 3 d. Mice were humanely euthanized when they were overwhelmed by tumor burden. All tumors and major organs were fixed in formalin and embedded in paraffin. Histopathological analysis was performed following routine hematoxylin and eosin (HE) staining on tissue sections. The tumor volume was calculated on the basis of the following formula: volume = $(\pi/6)$ LWH (L = length, W = width, H = height).

Statistical analysis

The Student *t*-test and the Fisher exact probability test were used for statistical analysis; a *P* value of less than 0.05 was considered significant.

RESULTS

Fascin expression in pancreatic cancer cell lines and the generation of fascin-overexpressing pancreatic cancer cells

Western blotting analysis was performed to investigate the expression of fascin in different human pancreatic cancer cell lines. Fascin protein was present at different

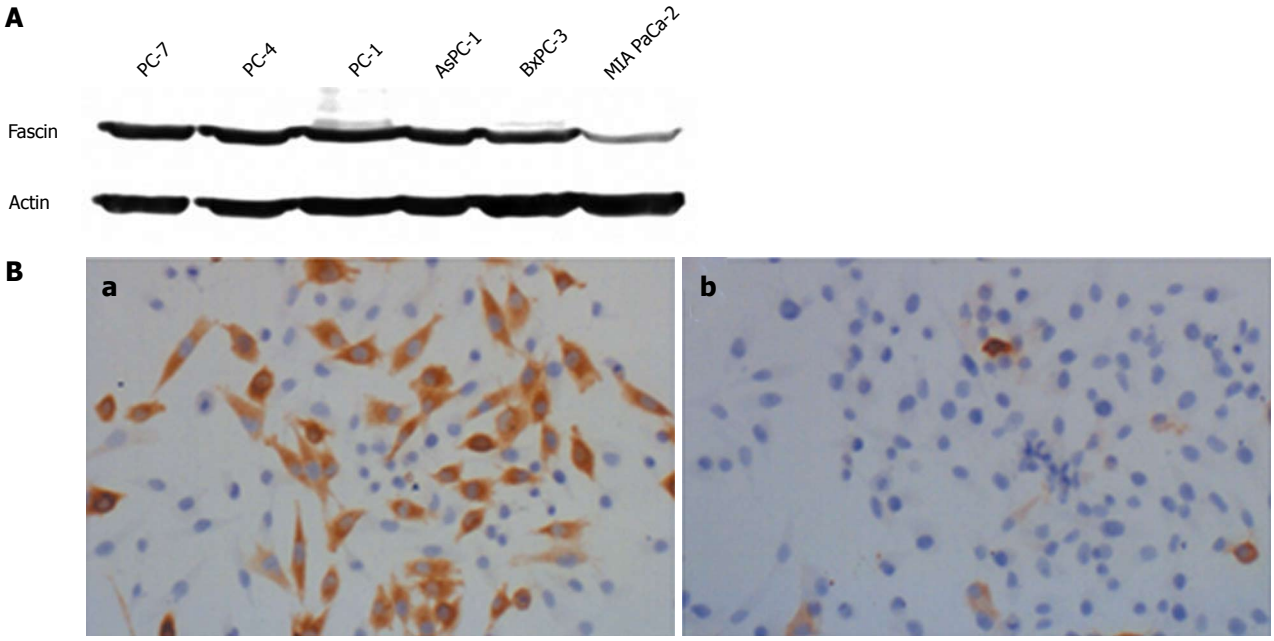

Figure 1 The selection and generation of fascin-overexpressing pancreatic cancer cells. A: Western blotting analysis of fascin expression in pancreatic cancer cell lines. Fascin protein was present in all pancreatic cancer cell lines at different expression levels. BxPC-3, AsPC-1, PC-1, PC-4 and PC-7 express fascin at a high level, whereas MIA PaCa-2 expresses fascin at a very low level. Actin served as a loading control; B: Immunohistochemical analysis of fascin expression in MIA PaCa-2 cells transfected with either pcDNA3-Fascin or pcDNA3-Vector. (a) MIA PaCa-2 Fascin represents the stable transfected fascin-expressing cell line; (b) MIA PaCa-2 Vector is the control cell line. Magnification is × 200.

expression levels in all of the tested pancreatic cancer cell lines. BxPC-3, AsPC-1, PC-1, PC-4 and PC-7 expressed fascin at a high level, whereas MIA PaCa-2 expressed fascin at a very low level (Figure 1A). Because MIA PaCa-2 cells endogenously express fascin at low levels, we chose this cell line to examine the effect of heterogeneous fascin expression on the biological properties of pancreatic cancer cells.

MIA PaCa-2 cells were transfected with either pcDNA3-Fascin or the pcDNA3-Vector and stable clones were selected by G418 treatment. MIA PaCa-2 Fascin cells and MIA PaCa-2 Vector cells were used for further analysis (Figure 1B).

Fascin overexpression induces alteration of cell morphology and cytoskeleton

There was an increase in membrane protrusions in the MIA PaCa-2 Fascin cells when compared with the control MIA PaCa-2 Vector cells. Morphologically, the MIA PaCa-2 Vector cells were more rounded and had fewer projections, whereas MIA PaCa-2 Fascin cells were polarized with elongated membrane projections. In MIA PaCa-2 Fascin cells, actin filaments were distributed as bundles in the cytoplasm that protruded into membrane projections, whereas the actin filaments in MIA PaCa-2 Vector cells were distributed in a diffuse manner (Figure 2A). This result was also visualized *via* immunofluorescence as an accumulation of actin filaments in a polarized manner in MIA PaCa-2 Fascin cells and as a diffuse distribution in MIA PaCa-2 Vector cells (Figure 2B).

Table 1 Cell cycle analysis of fascin-transfected MIA PaCa-2 cells (MIA PaCa-2 Fascin) and vector-transfected control MIA PaCa-2 cells (MIA PaCa-2-Vector) (mean ± SD)

	G1 (%)[a]	S (%)	G2 (%)
MiaPaCa-2-Fascin	74.67 ± 3.89	17.1 ± 4.16	8.23 ± 0.81
MiaPaCa-2-Vector	66 ± 3.01	22.83 ± 4.55	11.17 ± 1.66

Percentages of the total cell population in different phases of the cell cycle were determined. The mean values of three experiments are shown. ([a]$P < 0.05$).

Heterogeneous expression of fascin does not promote pancreatic cancer cell growth in vitro and in vivo

The growth curves of MIA PaCa-2 Fascin and MIA PaCa-2 Vector cells showed no significant difference between the two groups (Figure 3). Therefore, the heterogeneous expression of fascin does not seem to affect pancreatic cancer cell growth rate *in vitro*.

As shown in Table 1, fascin transfection induced an increase in G1 phase without a significant decrease in G2/M and S phases.

When transplanted into nude mice, both the MIA PaCa-2 Fascin and MIA PaCa-2 Vector cells developed solid tumor masses. The mean tumor volume from MIA PaCa-2 Fascin and MIA PaCa-2 Vector cells was 2.86 ± 2.24 cm^3 and 3.08 ± 1.16 cm^3, respectively. Tumors from fascin-transfected cells grew at a slightly slower rate in comparison with control tumors, but this difference was not significant ($P = 0.8439$). These results are in agreement with our *in vitro* experiments.

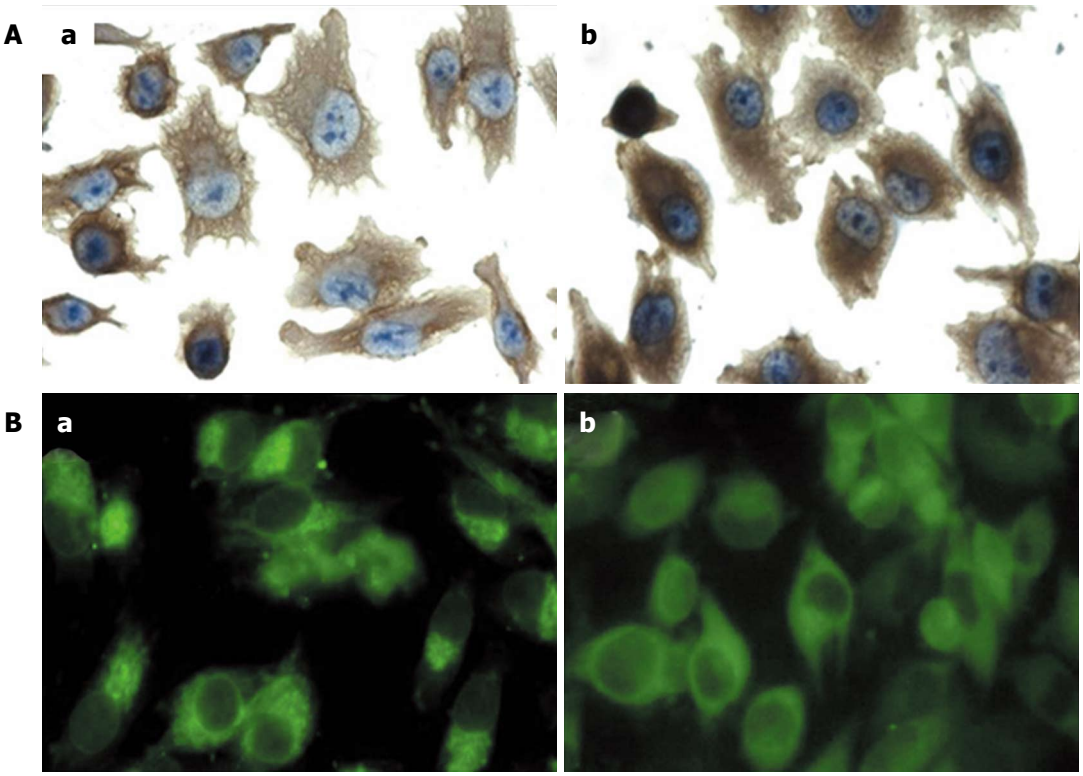

Figure 2 Fascin overexpression induces alteration of cell morphology and cytoskeleton. A: Immunohistochemical analysis of actin distribution in fascin-overexpressing cells and vector control cells (× 400). (a) MIA PaCa-2 Fascin cells were more polarized with elongated membrane projections. Actin filaments were distributed as bundles in the cytoplasm which protruded into membrane projections in MIA PaCa-2 Fascin cells. (b) MIA PaCa-2 Vector cells showed a diffuse actin distribution; B: Immunofluorescence analysis of actin distribution in fascin-overexpressing cells and vector control cells (× 400). (a) Actin accumulated in a polarized manner in MIA PaCa-2 Fascin cells, whereas (b) MIA PaCa-2 Vector cells demonstrated a diffuse actin distribution.

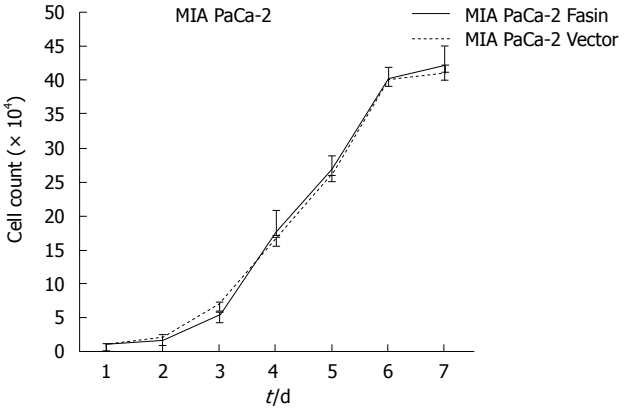

Figure 3 The growth curves of MIA PaCa-2 Fascin and MIA PaCa-2 Vector cells. There were no significant differences between the two groups.

Fascin promotes cell migration and inhibits cell aggregation

To investigate the effects of fascin on cell migration, *in vitro* wound healing assays were performed. After wounds were made for 20 h, the MIA PaCa-2 Fascin and MIA PaCa-2 Vector cells exhibited a cell reorientation response along the wounded edge margin and migrated into the wound area. MIA PaCa-2 Fascin cells repopulated the open space more efficiently than did MIA PaCa-2 Vector cells (Figure 4A).

Cell aggregation is an important factor that may critically affect tumor cell metastasis. We tested this using a hanging drop cell aggregation assay. Our results showed that the heterogeneous expression of fascin resulted in a reduction in aggregation when compared with vector control cells (Figure 4B).

Fascin promotes pancreatic cancer cell invasiveness in vitro and in vivo

To determine whether fascin promotes pancreatic cancer cell invasion, an *in vitro* invasion assay was performed using a Matrigel Invasion Chamber. Overexpression of fascin dramatically increased the cell invasive properties of the MIA PaCa-2 cells when compared with control MIA PaCa-2 cells (Figure 5A).

When transplanted into nude mice, the tumors developed from fascin-overexpressing MIA PaCa-2 Fascin cells grew in a more aggressive pattern, as 4 out of 6 of these tumors showed skin invasion, whereas only 1 of the control tumors exhibited skin invasion (Figure 5B and 5C).

DISCUSSION

We detected fascin expression in 6 pancreatic cancer cell lines (BxPC-3, AsPC-1, MIA PaCa-2 and 3 cell lines established by our laboratory: PC-1, PC-4 and PC-7). All of the cell lines expressed fascin at a relatively high

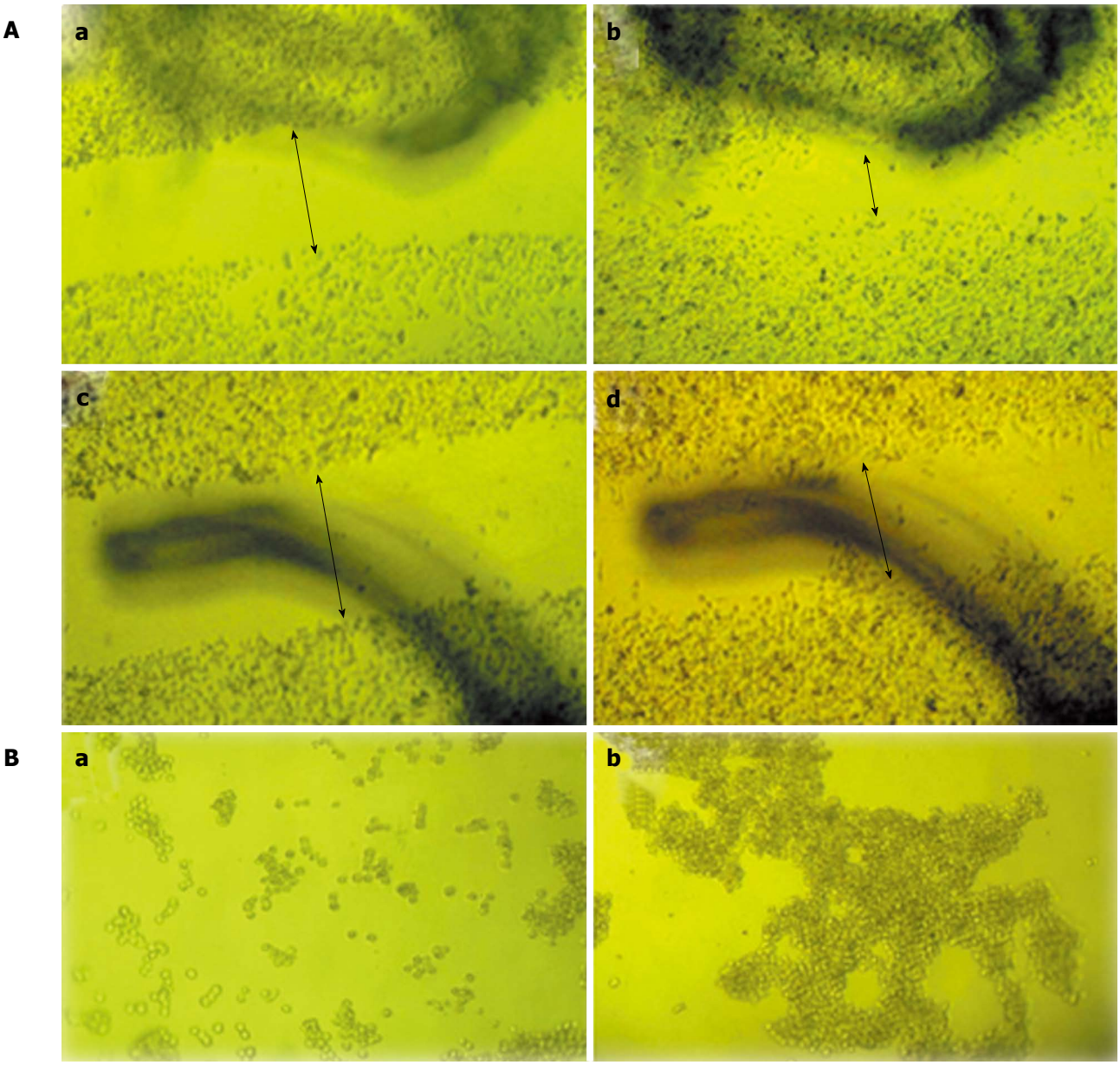

Figure 4 Fascin promotes cell migration and inhibits cell aggregation. A: The effect of fascin overexpression on the cell migration of MIA PaCa-2 cells. Images were taken at 0 h (a, c) and 20 h (b, d) (× 60). MIA PaCa-2 Fascin cells (a, b) repopulated the open space more efficiently than did MIA PaCa-2 Vector cells (c, d). The arrows in figure 4a (MIA PaCa-2-Fascin cells group) and figure 4c (MIA PaCa-2-Vector cells group) showed initial distances between two side of cells (0 h), and the arrow in figure 4b (MIA PaCa-2-Fascin cells group) was short than the one in figure 4d (MIA PaCa-2-Vector cells group) in 20 h, which showed the ability of cell migration increased in the state of fascin overexpression; B: The effect of fascin overexpression on the aggregation of MIA PaCa-2 cells. Images were taken at 12 h (× 60). The heterogeneous expression of fascin resulted in a reduction in aggregation compared with control cells. a: MIA PaCa-2 Fascin cells; b: MIA PaCa-2 Vector cells.

level, except MIA PaCa-2. This finding may indicate that fascin overexpression is a common event in pancreatic cancer, but the pathogenic effects of fascin are different among these cell lines. To elucidate the function of fascin in pancreatic cancer cells, we introduced a fascin-expression vector into MIA PaCa-2 cells and found that heterogeneous expression of fascin resulted in an increase in cell invasiveness and motility with a decrease in cell aggregation. The proliferation and cell cycle distribution of pancreatic cancer cells was not obviously affected by fascin overexpression. To our knowledge, this is the first study to ascertain the function of fascin by means of heterogeneous overexpression in pancreatic cancer cells.

Pancreatic cancer progresses rapidly and demonstrates strong invasion and early metastatic properties with poor prognosis. The characteristics of tumor progression, cell motility and invasiveness, result from a rearrangement of the cytoskeletal microfilaments that is modulated by several types of actin cross-linking proteins[19]. Among these molecules, fascin is implicated in the organization and persistence of filopodia, which plays an important role in cell-matrix adhesion, cell interactions and cell migration[20,21]. Additional evidence has shown fascin-overexpressing tumors to have increased invasive properties. In breast cancer cells, overexpression

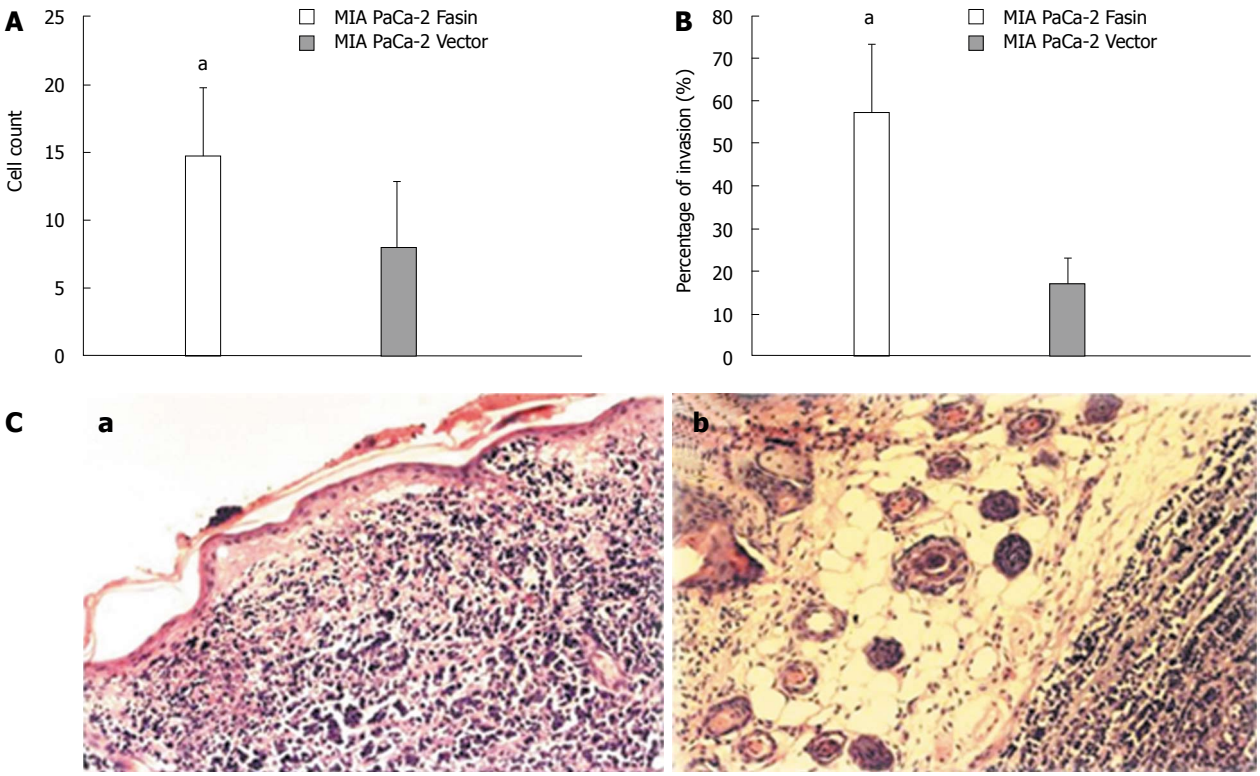

Figure 5 The effect of fascin overexpression on invasiveness of MIA PaCa-2 cells. A: Matrigel invasion assay showed that the overexpression of fascin dramatically increased cell invasive properties compared with control cells. ($^aP < 0.05$); B: *In vivo* invasion assay. Tumors from fascin-overexpressing MIA PaCa-2 Fascin cells showed an increase in skin invasion when compared with control MIA PaCa-2 Vector cells; C: Histological examination of skin invasion of tumors from MIA PaCa-2 Fascin cells and control MIA PaCa-2 cells. (a) Tumor from MIA PaCa-2 Fascin cells had skin invasion and (b) tumor from MIA PaCa-2 Vector cells showed no skin invasion (HE, × 150). HE: Hematoxylin and eosin.

of c-erbB2 resulted in an increase in fascin expression and tumor cell motility[22]. Also in gastric carcinoma, the outer edges of the tumors tended to have the most intense fascin staining in an immunohistochemical assay[13]. Jawhari *et al*[23] found that de novo expression of fascin in well-differentiated colon cancer cells increased cell migration through collagen type I - or IV-coated filters, and the cells showed a significant increase in dynamic membrane activity. In addition, in esophageal squamous cell carcinoma the down-regulation of endogenous fascin by RNA interference resulted in a dramatic decrease in cell invasiveness[24]. In this study, we found that heterogeneous overexpression of fascin in pancreatic cancer cells resulted in an increase in cell motility and invasiveness. In fascin-overexpressing cells, there were more membrane protrusions, and the actin filaments were arranged as bundles in the cytoplasm which protruded into the membrane projections. In contrast, the control cells were rounded with diffusely distributed actin filaments and fewer projections. Thus, it seems likely that the rearrangement of the actin cytoskeleton induced by fascin overexpression in pancreatic cancer cells promoted their motility and invasion, which resulted in a more aggressive phenotype.

Cancer cell adhesion and migration are distinct but related events in the process of cancer progression, and cell dissociation is one of the limiting steps during the course of cancer cell migration. Heterogeneous overexpression of fascin in pancreatic cancer cells resulted in an obvious decrease in cell-cell adhesion, as shown in the aggregation assay. To date, there are few reports on the role of fascin in cell-cell adhesion. Ectopic expression of fascin in rat Con8 cells disrupted the dexamethasone-induced formation of tight junctions and adherent junctions by preventing the recruitment of occludin and β-catenin to the site of cell-cell contact, which suggested that fascin was a negative regulator of cell-cell interactions[25]. Another study demonstrated that fascin competed with E-cadherin for an association with β-catenin *in vitro*[26], and it is conceivable that fascin plays a role in modulating cell adhesion. In contrast, a study in colon cancer cells did not find an effect of fascin on the E-cadherin-β-catenin association and distribution[27]. Thus, the molecular mechanism of fascin involved in cell-cell adhesion still needs to be further explored.

In this study, the overexpression of fascin had no obvious effect on pancreatic cancer cell proliferation, which is in contrast to Jawhari *et al*[23], who reported that fascin promoted the proliferation of colon cancer cells. In lung carcinomas, highly fascin-positive tumors had a high Ki-67 index[14]. However, in colorectal adenoma, fascin and ki-67 were inversely correlated[11]. The reasons for these divergent findings are currently unknown and may be related to the differences in growth-regulating signaling pathways. As a poorly differentiated pancreatic cancer cell line with the shortest doubling time compared

to the other 11 pancreatic cancer cell lines[28], the MIA PaCa-2 cell line may have growth-regulating signaling pathways which are less dependent on fascin expression.

In summary, our study showed that overexpression of fascin promoted pancreatic cancer cell dissociation, migration and invasion, indicating its usefulness as a pancreatic cancer gene therapy target.

ACKNOWLEDGMENTS

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COMMENTS

Background

Pancreatic cancer is one of the most devastating human malignancies, with an overall 5 year survival rate of 5% and a median survival time of 6 mo, and the major biological hallmarks of this disease are its early and aggressive local invasion and metastasis. Fascin is associated with cell movement and was identified to show the greatest change between pancreatic cancer and normal pancreas samples.

Research frontiers

Fascin expression is often absent in normal epithelial cells, and its expression is upregulated in several human neoplasms. Fascin overexpression is often correlated with an invasive tumor phenotype, poor prognosis and decreased disease-free survival. The role of fascin in the malignant behavior of pancreatic cancer remains unknown. In this study, the authors demonstrate that the overexpression of fascin could be a potential mechanism for migration and invasion in pancreatic cancer.

Innovations and breakthroughs

Recent reports have highlighted the importance of fascin in many types of cancer. This is the first study to verify that fascin is over-expressed in pancreatic cancer cells and that it promotes tumor migration and invasion. Furthermore, our *in vitro* and *in vivo* studies would suggest that this protein may be a positive factor of invasion and metastasis in this cancer.

Applications

By understanding fascin's overexpression and whether it induces migration and invasion, the findings of this study may represent a future strategy for therapeutic intervention in the treatment of patients with pancreatic cancer.

Terminology

The cytoskeletal protein, fascin, is an actin-bundling protein that plays a role in cell matrix adhesion, cell interaction and migration. Its overexpression has been reported in many types of tumors, but its function in pancreatic cancer is still unknown.

Peer review

The authors explored the role of Fascin during the progression of pancreatic cancer in pancreatic cancer cell lines and a mouse model. The key findings were that fascin promotes cancer cell migration, invasion and scattering leading to a more aggressive phenotype. Fascin overexpression did not result in increased cell cycle and proliferation which is in contrast to other tumor types, i.e., colon cancer. In general, the experiments are well explained and executed.

REFERENCES

1 **Lu Z**, Hu L, Evers S, Chen J, Shen Y. Differential expression profiling of human pancreatic adenocarcinoma and healthy pancreatic tissue. *Proteomics* 2004; **4**: 3975-3988
2 **Yamaguchi H**, Inoue T, Eguchi T, Miyasaka Y, Ohuchida K, Mizumoto K, Yamada T, Yamaguchi K, Tanaka M, Tsuneyoshi M. Fascin overexpression in intraductal papillary mucinous neoplasms (adenomas, borderline neoplasms, and carcinomas) of the pancreas, correlated with increased histological grade. *Mod Pathol* 2007; **20**: 552-561
3 **Maitra A**, Iacobuzio-Donahue C, Rahman A, Sohn TA, Argani P, Meyer R, Yeo CJ, Cameron JL, Goggins M, Kern SE, Ashfaq R, Hruban RH, Wilentz RE. Immunohistochemical validation of a novel epithelial and a novel stromal marker of pancreatic ductal adenocarcinoma identified by global expression microarrays: sea urchin fascin homolog and heat shock protein 47. *Am J Clin Pathol* 2002; **118**: 52-59
4 **Maitra A**, Adsay NV, Argani P, Iacobuzio-Donahue C, De Marzo A, Cameron JL, Yeo CJ, Hruban RH. Multicomponent analysis of the pancreatic adenocarcinoma progression model using a pancreatic intraepithelial neoplasia tissue microarray. *Mod Pathol* 2003; **16**: 902-912
5 **Iacobuzio-Donahue CA**, Ashfaq R, Maitra A, Adsay NV, Shen-Ong GL, Berg K, Hollingsworth MA, Cameron JL, Yeo CJ, Kern SE, Goggins M, Hruban RH. Highly expressed genes in pancreatic ductal adenocarcinomas: a comprehensive characterization and comparison of the transcription profiles obtained from three major technologies. *Cancer Res* 2003; **63**: 8614-8622
6 **Kureishy N**, Sapountzi V, Prag S, Anilkumar N, Adams JC. Fascins, and their roles in cell structure and function. *Bioessays* 2002; **24**: 350-361
7 **Adams JC**. Fascin protrusions in cell interactions. *Trends Cardiovasc Med* 2004; **14**: 221-226
8 **Swierczynski SL**, Maitra A, Abraham SC, Iacobuzio-Donahue CA, Ashfaq R, Cameron JL, Schulick RD, Yeo CJ, Rahman A, Hinkle DA, Hruban RH, Argani P. Analysis of novel tumor markers in pancreatic and biliary carcinomas using tissue microarrays. *Hum Pathol* 2004; **35**: 357-366
9 **Tong GX**, Yee H, Chiriboga L, Hernandez O, Waisman J. Fascin-1 expression in papillary and invasive urothelial carcinomas of the urinary bladder. *Hum Pathol* 2005; **36**: 741-746
10 **Yoder BJ**, Tso E, Skacel M, Pettay J, Tarr S, Budd T, Tubbs RR, Adams JC, Hicks DG. The expression of fascin, an actin-bundling motility protein, correlates with hormone receptor-negative breast cancer and a more aggressive clinical course. *Clin Cancer Res* 2005; **11**: 186-192
11 **Hashimoto Y**, Skacel M, Lavery IC, Mukherjee AL, Casey G, Adams JC. Prognostic significance of fascin expression in advanced colorectal cancer: an immunohistochemical study of colorectal adenomas and adenocarcinomas. *BMC Cancer* 2006; **6**: 241
12 **Wen YH**, Yee H, Goswami S, Shukla PS. Fascin expression in serous tumors of ovary correlates with aggressiveness of malignancy. *Int J Gynecol Pathol* 2009; **28**: 187-192
13 **Hashimoto Y**, Shimada Y, Kawamura J, Yamasaki S, Imamura M. The prognostic relevance of fascin expression in human gastric carcinoma. *Oncology* 2004; **67**: 262-270
14 **Pelosi G**, Pastorino U, Pasini F, Maissoneuve P, Fraggetta F, Iannucci A, Sonzogni A, De Manzoni G, Terzi A, Durante E, Bresaola E, Pezzella F, Viale G. Independent prognostic value of fascin immunoreactivity in stage I nonsmall cell lung cancer. *Br J Cancer* 2003; **88**: 537-547
15 **Zigeuner R**, Droschl N, Tauber V, Rehak P, Langner C. Biologic significance of fascin expression in clear cell renal cell carcinoma: systematic analysis of primary and metastatic tumor tissues using a tissue microarray technique. *Urology* 2006; **68**: 518-522
16 **Darnel AD**, Behmoaram E, Vollmer RT, Corcos J, Bijian K, Sircar K, Su J, Jiao J, Alaoui-Jamali MA, Bismar TA. Fascin regulates prostate cancer cell invasion and is associated with metastasis and biochemical failure in prostate cancer. *Clin Cancer Res* 2009; **15**: 1376-1383
17 **Lee LT**, Huang YT, Hwang JJ, Lee AY, Ke FC, Huang CJ, Kandaswami C, Lee PP, Lee MT. Transinactivation of the epidermal growth factor receptor tyrosine kinase and focal adhesion kinase phosphorylation by dietary flavonoids: effect on invasive potential of human carcinoma cells. *Biochem Pharmacol* 2004; **67**: 2103-2114

18 **Singh AP**, Moniaux N, Chauhan SC, Meza JL, Batra SK. Inhibition of MUC4 expression suppresses pancreatic tumor cell growth and metastasis. *Cancer Res* 2004; **64**: 622-630

19 **Tseng Y**, Kole TP, Lee JS, Fedorov E, Almo SC, Schafer BW, Wirtz D. How actin crosslinking and bundling proteins cooperate to generate an enhanced cell mechanical response. *Biochem Biophys Res Commun* 2005; **334**: 183-192

20 **Zhang J**, Fonovic M, Suyama K, Bogyo M, Scott MP. Rab35 controls actin bundling by recruiting fascin as an effector protein. *Science* 2009; **325**: 1250-1254

21 **Hashimoto Y**, Skacel M, Adams JC. Roles of fascin in human carcinoma motility and signaling: prospects for a novel biomarker? *Int J Biochem Cell Biol* 2005; **37**: 1787-1804

22 **Grothey A**, Hashizume R, Ji H, Tubb BE, Patrick CW, Yu D, Mooney EE, McCrea PD. C-erbB-2/ HER-2 upregulates fascin, an actin-bundling protein associated with cell motility, in human breast cancer cell lines. *Oncogene* 2000; **19**: 4864-4875

23 **Jawhari AU**, Buda A, Jenkins M, Shehzad K, Sarraf C, Noda M, Farthing MJ, Pignatelli M, Adams JC. Fascin, an actin-bundling protein, modulates colonic epithelial cell invasiveness and differentiation in vitro. *Am J Pathol* 2003; **162**: 69-80

24 **Xie JJ**, Xu LY, Zhang HH, Cai WJ, Mai RQ, Xie YM, Yang ZM, Niu YD, Shen ZY, Li EM. Role of fascin in the proliferation and invasiveness of esophageal carcinoma cells. *Biochem Biophys Res Commun* 2005; **337**: 355-362

25 **Wong V**, Ching D, McCrea PD, Firestone GL. Glucocorticoid down-regulation of fascin protein expression is required for the steroid-induced formation of tight junctions and cell-cell interactions in rat mammary epithelial tumor cells. *J Biol Chem* 1999; **274**: 5443-5453

26 **Tao YS**, Edwards RA, Tubb B, Wang S, Bryan J, McCrea PD. beta-Catenin associates with the actin-bundling protein fascin in a noncadherin complex. *J Cell Biol* 1996; **134**: 1271-1281

27 **Hahn-Strömberg V**, Edvardsson H, Bodin L, Franzén L. Disturbed expression of E-cadherin, beta-catenin and tight junction proteins in colon carcinoma is unrelated to growth pattern and genetic polymorphisms. *APMIS* 2008; **116**: 253-262

28 **Sipos B**, Möser S, Kalthoff H, Török V, Löhr M, Klöppel G. A comprehensive characterization of pancreatic ductal carcinoma cell lines: towards the establishment of an in vitro research platform. *Virchows Arch* 2003; **442**: 444-452

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ORIGINAL ARTICLE

Expression and localization of paxillin in rat pancreas during development

Jing Guo, Li-Jie Liu, Li Yuan, Ning Wang, Wei De

Jing Guo, Li Yuan, Ning Wang, Wei De, Department of Biochemistry and Molecular Biology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China
Li-Jie Liu, Department of Physiology, Southeast University, Nanjing 210009, Jiangsu Province, China
Author contributions: Guo J and Liu LJ performed the majority of the experiments; Yuan L and Wang N provided vital reagents and analytical tools and were also involved in editing the manuscript; De W designed the study and wrote the manuscript; all authors read and approved the final manuscript.
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Correspondence to: Wei De, MD, PhD, Professor of Molecular Biology, Department of Biochemistry and Molecular Biology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China. dewei@njmu.edu.cn
Telephone: +86-25-86862728 Fax: +86-25-86862728
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Abstract

AIM: To investigate the expression and localization of paxillin in rat pancreas during development.

METHODS: Pancreata from Sprague Dawley rat fetuses, embryos, young animals, and adult animals were used in this study. Expression levels of paxillin in pancreata of different development stages were detected by reverse transcription polymerase chain reaction and Western blotting. To identify the cell location of paxillin in the developing rat pancreas, immunohistochemistry and double-immunofluorescent staining were performed using antibodies for specific cell markers and paxillin, respectively.

RESULTS: The highest paxillin mRNA level was detected at E15.5 (embryo day 15.5) following a decrease in the later developmental periods ($P < 0.05$ *vs* E18.5, P0 and adult, respectively), and a progressively increased paxillin protein expression through the transition from E15.5 to adult was detected. The paxillin positive staining was mainly localized in rat islets of Langerhans at each stage tested during pancreas development.

CONCLUSION: The dynamic expression of paxillin in rat pancreas from different stages indicates that paxillin might be involved in some aspects of pancreatic development.

Key words: Pancreas development; Islet remodeling; Paxillin; Cell adhesion; Migration

Peer reviewers: Parviz M Pour, Professor of Pathology, Member of UNMC/Eppley Cancer Center, 98168 Nebraska Med Center, Omaha, NE 68198-680, United States; Raffaele Pezzilli, MD, Department of Internal Medicine and Gastroenterology, Sant' Orsola-Malpighi Hospital, Via Massarenti, 9, Bologna 40138, Italy; Mukaddes Esrefoglu, Dr., Professor, Department of Histology and Embryology, Inonu University, 44280 Malatya, Turkey

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INTRODUCTION

Development of the endocrine and exocrine pancreas involves a complex process of cell differentiation that ultimately gives rise to four distinct hormone producing cell types (α, β, δ, PP) and two kinds of enzyme secreting cells (acinar and ductal cells)[1]. Organogenesis of the pancreas is a highly coordinated process. Morphologic development of the mouse pancreas first appears at E9.5. At approximately E16 the islet progenitor cells leave the

contiguous epithelium, migrate through the adjacent extracellular matrix (ECM) into the surrounding mesenchyme, and aggregate to form the islets of Langerhans[2,3]. The islets are not fully formed until shortly before birth in E18-E19, and undergo further remodeling and maturation for 2-3 wk after birth[4]. Thus, the developing pancreas presents a challenge for developmental biologists because of the complex morphogenetic processes underlying the development of this organ.

The factors that control pancreatic organogenesis and tissue maintenance remain unclear. Of particular interest are the ECM and their receptors, integrins, which exert a profound role during development controlling morphogenetic decisions and maintaining homeostasis during adulthood. Progression in islet cell development is accompanied by, and dependent upon, cell adhesion *via* β1 integrin and its respective α-subunits. The β1 family of integrins play critical roles in islet cell architecture, development, integrity and function[5].

Paxillin interacts directly with several focal adhesion proteins including vinculin, talin, and integrin β1[6,7]. A principal function for paxillin is in the integration and dissemination of signals from integrins and growth factor receptors to provide efficient cellular migration[8]. Paxillin is an important mediator of signal cross-talk in the complex multistep process of net cellular movement through its phosphorylation and multipotent associations[9-12], and functions as an adaptor protein coordinating the activities of many focal adhesion proteins. Thus, paxillin is in a position to play a role in the integration and regulation of adhesion and signaling, yet little is known regarding its function during embryogenesis[13]. Given that it mediates integrin signal transduction, it might be expected that paxillin may be involved in numerous aspects of cell behavior and development in the pancreas. To the best of our knowledge, no study has investigated the relationship between paxillin expression and pancreas development, and the expression of paxillin during pancreatic development in rats is poorly understood. Knowledge of the regional and temporal expression of paxillin will be useful in understanding its potential role in pancreatic development. Therefore, we examined the expression of paxillin in rat pancreas during development.

MATERIALS AND METHODS

Animals and preparation of rat pancreatic tissue

Sprague-Dawley (SD) rats were purchased from the Animal Center of Nanjing Medical University (Nanjing, China). SD rats (2:1, male:female) were mated overnight. At noon the next day, if a vaginal plug was discovered, it was considered as Day 0.5 of gestation (E0.5). Embryos were removed at E12.5, E15.5 and E18.5 from the uterus of pregnant rats, which were sacrificed by cervical dislocation. Pancreata from E15.5 and E18.5 rat embryos were isolated according to their specific vacuolated morphology, as previously described[14], under a stereomicroscope. Rat pancreata at postnatal (P) days 0, 7, 14, 21 and from adults, were directly isolated by the unaided eye. All experiments were conducted in accordance with the Chinese Law for Animal Protection and were approved by the local animal care committee. Five rats were used at each age stage. Dissected tissues were immediately rinsed 3 times with phosphate buffered saline (PBS) to remove serum proteins, and fixed with 4% paraformaldehyde in PBS overnight for histology, or frozen in liquid nitrogen for RNA and protein isolation.

Immunohistochemistry

Pancreata from E15.5, E18.5, P0, P14, P21, and nonpregnant adult rats were fixed with 4% paraformaldehyde in PBS overnight and embedded in paraffin. Pancreata were cut into 5-μm sections and mounted on gelatin/chrome alum-coated glass slides. Following deparaffinization, the presence of paxillin and insulin was determined immunohistochemically. To expose antigenic sites for paxillin/insulin, dewaxed sections were heated four times to 95 ℃ in a 600 W microwave oven maintained for 5 min and allowed to cool for 20 min. Endogenous peroxidase activity was then eliminated by incubation with 0.5% (v/v) hydrogen peroxide solution in absolute methanol for 15 min at 20 ℃. Non-specific protein binding was eliminated by incubation with 10% non-fat dry milk in PBS for 1 h at 20 ℃. Sections were then incubated with a polyclonal antibody (sc-7336; Santa Cruz Biotechnology) against paxillin or insulin (sc-9168; Santa Cruz Biotechnology) at a dilution of 1:200 and 1:500, respectively, for 18 h at 4 ℃. Incubation for 1 h with horseradish peroxidase conjugated secondary antibody (1:500 dilutions) at room temperature followed. The antigen-antibody complex was then visualized by incubating the sections with 3, 3'-diaminobenzidine solution in the dark for 3 min. Sections counterstained with hematoxylin were dehydrated, and coverslipped. Images were taken at a magnification of × 400. Controls were processed by omitting the primary antibody in the immunolabeling procedure.

Double fluorescence immunohistochemistry

The paraffin sections were deparaffinized in xylene and rehydrated in graded ethanol and distilled water. The nonspecific binding sites were blocked in 1% bovine serum albumin for 30 min. For paxillin and amylase or glucagon double immunofluorescence, the goat anti- paxillin primary polyclonal antibody was applied and revealed using fluorescein isothiocyanate-labeled rabbit anti-goat IgG (1:400, sc-2777, Santa Cruz Biotechnology). Mouse anti-amylase primary polyclonal antibody (1:500, sc-45667; Santa Cruz Biotechnology) or mouse anti-glucagon primary polyclonal antibody (1:1000, G2654; Sigma Aldrich) was then applied and revealed by cy3-labeled anti-mouse IgG (1:400, AP192C; Chemicon International, Inc. Temecula, CA, United States). Sections were placed in gel mounted aqueous mounting medium (G0918; Sigma, St. Louis, United States) with a cover glass, and examined under an Olympus BX51 Research Microscope (Olympus Optical, Tokyo, Japan). To rule out cross-reactivity in this staining system, the controls used were: first, single stain-

Table 1 mRNA expression of genes related to paxillin in the developing rat pancreas

Gene symbol	Probeset ID	E12.5		E15.5		E18.5		P0		Adult	
Integrinβ1	1368819_at	748.3	P	3277.2	P	3847.5	P	1055.6	P	427.5	P
Integrinβ4	1368612_at	58.2	A	295.3	P	542.6	P	149.4	P	115.5	P
Integrinβ5	1370801_at	25.4	A	49.5	P	56.3	P	5.2	A	2.8	A
Vinculin	1375538_at	168.7	P	1532.5	P	1790.7	P	141.9	P	23.1	P
ILK	1387777_at	634.5	P	1388.5	P	1449.5	P	583.3	P	122.8	P
Clathrin	1398842_at	1040.0	P	2918.2	P	3682.7	P	1306.9	P	429.0	P
Paxillin	1371664_at	298.8	P	997.5	P	847.0	P	285.3	P	62.4	P
Actopaxin	1370266_at	57.7	A	425.6	P	212.5	P	42.5	P	10.2	A

The Probeset IDs were the reference number for the probes of the Affymetrix oligonucleotide microarray (RAE230A). P: Present; A: Absent.

ing with the alternative secondary antibody and second, staining in the absence of primary antibody. In neither case was staining detectable. Images were taken at a magnification of × 400.

Preparation of protein samples

The pancreata was homogenized in a detergent lysis buffer containing 8 mol/L urea, 2% CHAPS, 40 mmol/L Tris, 65 mmol/L DTT and 2% IPG buffer. The lysate was then centrifuged at 15000 *g* for 1 h at 4 ℃. The total protein concentration of each sample was analyzed using a modified Bradford assay. All samples were stored at -80 ℃ prior to the electrophoresis.

Western blotting analysis

An equal amount of protein sample (40 μg) from each time point was resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). After transfer, the membrane was blocked with 5% fat-free milk in Tris-buffered saline and 0.05% Tween 20 overnight at 4 ℃. Primary antibodies were incubated with the membrane as described above and detected by peroxidase-linked rabbit anti-goat conjugates (Santa Cruz Biotechnology). Densitometric quantification of bands at subsaturating levels was performed using the Syngene-tool gel analysis software (Syngene, Cambridge, United Kingdom). Loading controls of presumably constantly expressed proteins such as β-tubulin were used; however, their variability and increase in development precluded their use[5]. For negative controls, the primary antibody was omitted.

RNA extraction, reverse transcription-polymerase chain reaction

Total RNA was extracted from the pancreata at each time point with TRIZOL reagent (Invitrogen Life Technologies, Burlington, Ontario, Canada), according to the manufacturer's instructions. The quality of the RNA was verified by agarose gel electrophoresis using ethidium bromide staining. For each polymerase chain reaction (PCR), 2 μg DNA-free total RNA with oligo (deoxythymidine) primers and reverse transcriptase were used. PCR was performed in 25-μL reactions containing 25 ng of cDNA, 0.2 nmol of each primer pair, and 0.3 μL of *Taq* DNA polymerase. PCR was carried out in a T-gradient Biometra PCR thermal cycler (Montreal Biotech Inc., Kirkland, Quebec, Canada) to determine the annealing temperature for paxillin primers. The primer pairs used were as follows: paxillin, forward: 5'-GGAGCAGAACGACAAGCC-3', reverse: 5'-GCACAGAGCCCAGGAGA-3' (256 bp); 18S rRNA, forward: 5'-ACGAACCAGAGCGAAAGC-3', reverse: 5'-GGACATCTAAGGGCATCACAG-3' (514 bp). PCR conditions were as follows: 2 min at 94 ℃ for hot start, followed by up to 35 cycles of 94 ℃ for 30 s, 53.4 ℃ for 30 s, and 72 ℃ for 45 s, with a final extension of 5 min at 72 ℃. To estimate the linear range of the nested reactions, we analyzed the PCR products at 10, 15, 20, 25, 30, and 35 cycles. The amplified products were analyzed on 1% agarose gels and visualized by ethidium bromide staining. The data were normalized by 18S rRNA.

cRNA probe generation and hybridization to Affymetrix microarray chips

Total RNA samples were used to generate cRNA probes by two rounds of transcription. A poly (dT) primer (with its 5' end carrying T7 promoter sequence) was used to synthesize cDNA from total RNA. The cDNA were used to amplify cRNA using T7 polymerase. The cRNA product from this first round amplification was then used to generate more cDNA by random priming, with the 3' end carrying a T7 promoter sequence. This cDNA was used to transcribe biotinylated cRNA, which was used to hybridize to the RAE 230A microarrays produced by Affymetrix.

Statistical analysis

Analysis of the experimental data was performed using PD Quest 7.0 software and the paired Student *t*-test. $P < 0.05$ was considered statistically significant. Data are presented as the mean ± SD.

RESULTS

Paxillin mRNA expression in the process of pancreatic development

The mRNA expression levels of paxillin in the whole

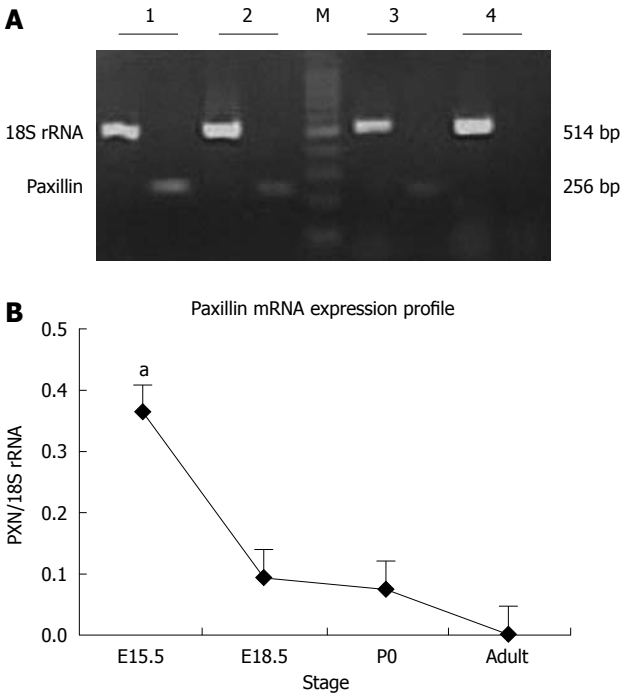

Figure 1 Paxillin mRNA expression level in the developing rat pancreas measured by reverse transcriptional polymerase chain reaction. A: From embryonic day (E) 15.5 to adulthood, paxillin mRNA expression in pancreas was highest at E15.5, following a dramatic decrease. Paxillin mRNA was almost undetectable in the adult pancreas. 1: E15.5; 2: E18.5; 3: P0; 4: Adult; M: Marker; B: Paxillin mRNA expression was analyzed and normalized to 18S rRNA. Results are indicated in percentages above the 18S rRNA value and are representative of three independent experiments. Compared to E15.5, the level of paxillin mRNA was lower at E18.5, P0 and adult ($^aP < 0.05$ *vs* E18.5, P0 and adult, respectively).

pancreas of the developing rat were examined through Affymetrix oligonucleotide microarray (RAE230A) (Table 1) and reverse transcription-PCR (Figure 1). As shown in Figure 1, the highest paxillin mRNA expression level was observed at E15.5, followed by decreased mRNA level of paxillin from E18.5 to adult. At the same time, mRNA expression of genes related to paxillin was also detected by Affymetrix oligonucleotide microarray (RAE230A), such as actopaxin, ILK, clathrin, vinculin, and Integrinβ1, as shown in Table 1. Absolute signal values of a selection of genes expressed in E12.5, E15.5, E18.5, P0 and adult rat pancreata were given. The highest expression level of these genes was almost detected at E15.5 (paxillin and actopaxin) or E18.5 (Integrinβ1, Integrinβ4, Integrinβ5, vinculin, ILK and clathrin). Expression profile of these mRNAs was similar to paxillin.

Regional localization of paxillin protein in rat pancreas at different developmental stages

To investigate the spatiotemporal expression of paxillin within the rat pancreas through fetal to postnatal life, we examined paxillin expression through immunohistochemistry (Figure 2). We found that paxillin maintained expression in the pancreas from E18.5 to adult, and at P14 and P21 paxillin was mainly localized in rat islets of Langerhans. Furthermore, double immunofluorescence

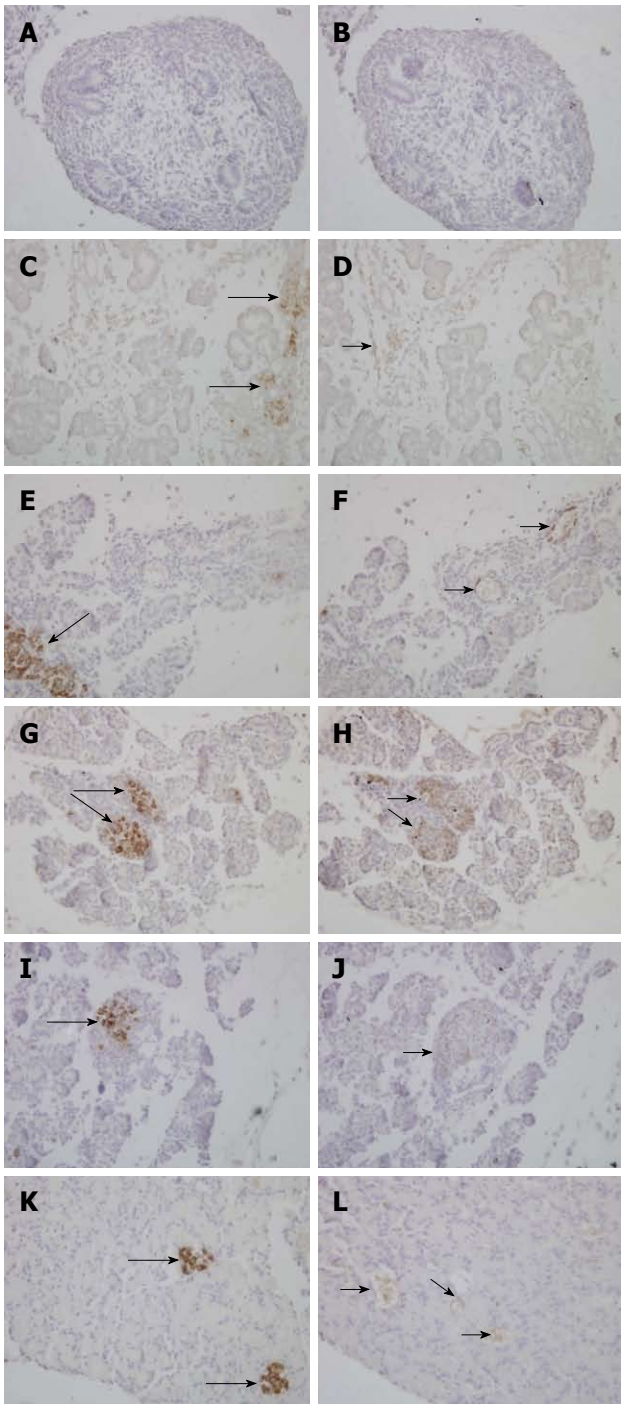

Figure 2 Immunohistochemical analysis of insulin and paxillin in the serial sections of the rat pancreas at E15.5 (A, B), E18.5 (C, D), P0 (E, F), P14 (G, H), P21 (I, J) and adult (K, L). Adjacent pancreatic sections from six developmental stages were stained with antibodies against insulin (left lane) and paxillin (right lane), respectively. We acquired images using an OLYMPUS DP70 digital camera. Strong cytoplasmic staining was observed for insulin (long arrows) for five stages except E15.5. Immunolocalization for the paxillin revealed a sporadic positive staining (short arrows) in the pancreas. In E18.5 and P0 rats, some cells in the pancreas, but not in islets, were stained. As shown in G-J, at P14 and P21 paxillin was mainly localized in islets. All magnifications are × 400.

was used to detect expression of paxillin and amylase (Figure 3) or glucagon (Figure 4). As shown in Figure 3, although little co-expression of paxillin and amylase was

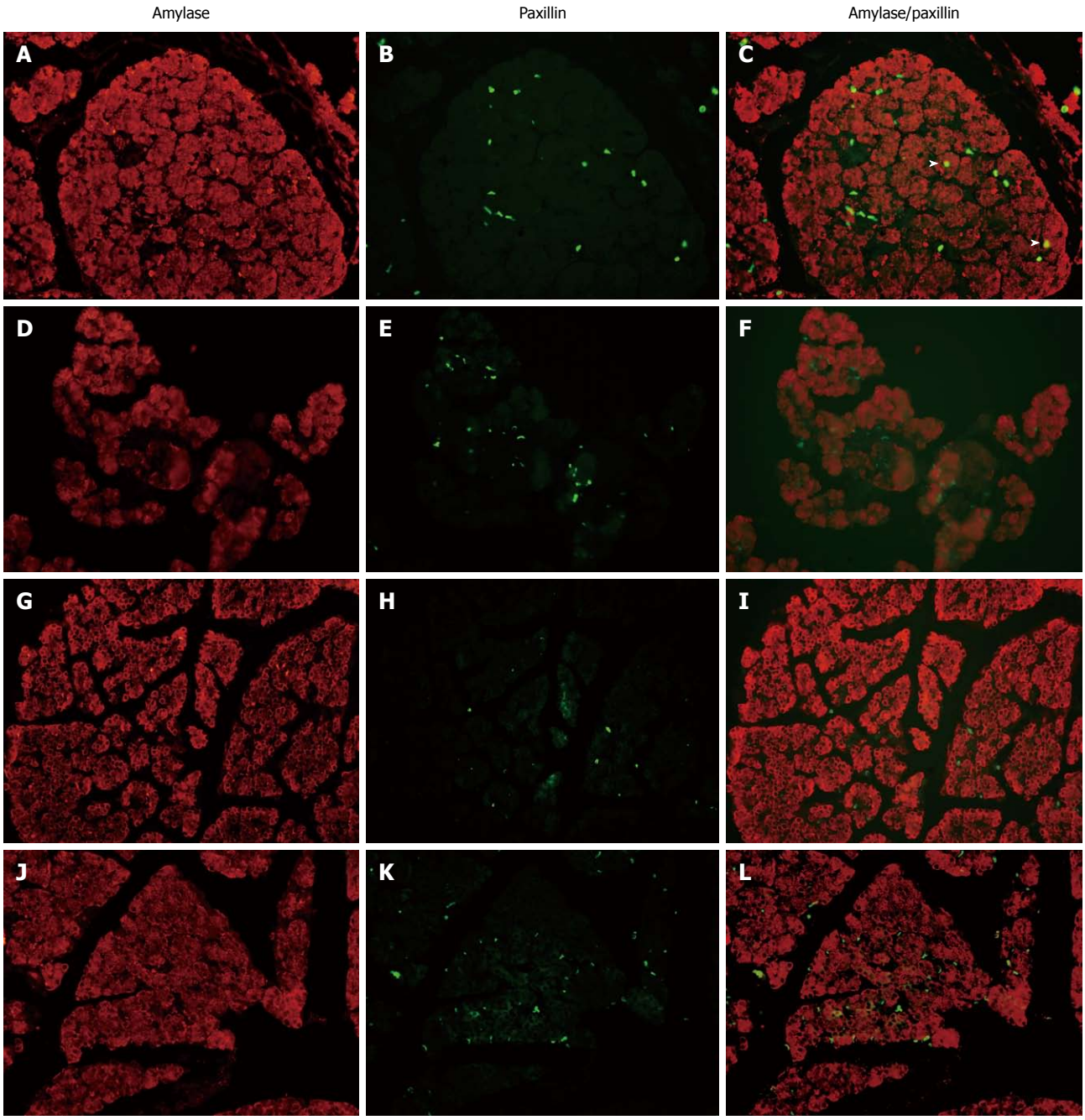

Figure 3 Immunofluorescent localization of paxillin and amylase in the pancreas of P0, P7, P21 and adult rats. The paxillin antibody was detected with an fluorescein isothiocyanate (green)-labeled secondary antibody and the amylase antibody was detected with a Cy3 (red)-labeled secondary antibody. Overlap between paxillin (green) and amylase (red) labeling is indicated by arrowheads. Original magnification 400 ×. A-C: P0; D-F: P7; G-I: P21; J-L: Adult.

detected, paxillin could be detected in the exocrine portion during development as has been reported previously[15]. As shown in Figure 4, although little co-expression of paxillin and glucagon was detected, paxillin positive cells could be found in the center of islets of Langerhans from E18.5. To the best of our knowledge, there have been no reports concerning the expression of paxillin in rat islets of Langerhans.

Paxillin protein expression in the process of pancreatic development

Examining the abundance of paxillin at the protein level during rat pancreatic development, from embryonic to postnatal life in whole pancreata by Western blotting analysis (Figure 5), revealed that there exist two isoforms of paxillin protein at 68 KD in pancreas during the developmental process, which was in agreement with previous reports[13]. During embryonic and neonatal development phases, only the smaller isoform was detected, while the larger isoform was detected only after birth. Interestingly, in the adult pancreas, the two isoforms could both be identified. Paxillin began to be expressed at E15.5, and significantly increased after birth, with a 10 fold increase at birth. The differential expression pattern of two iso-

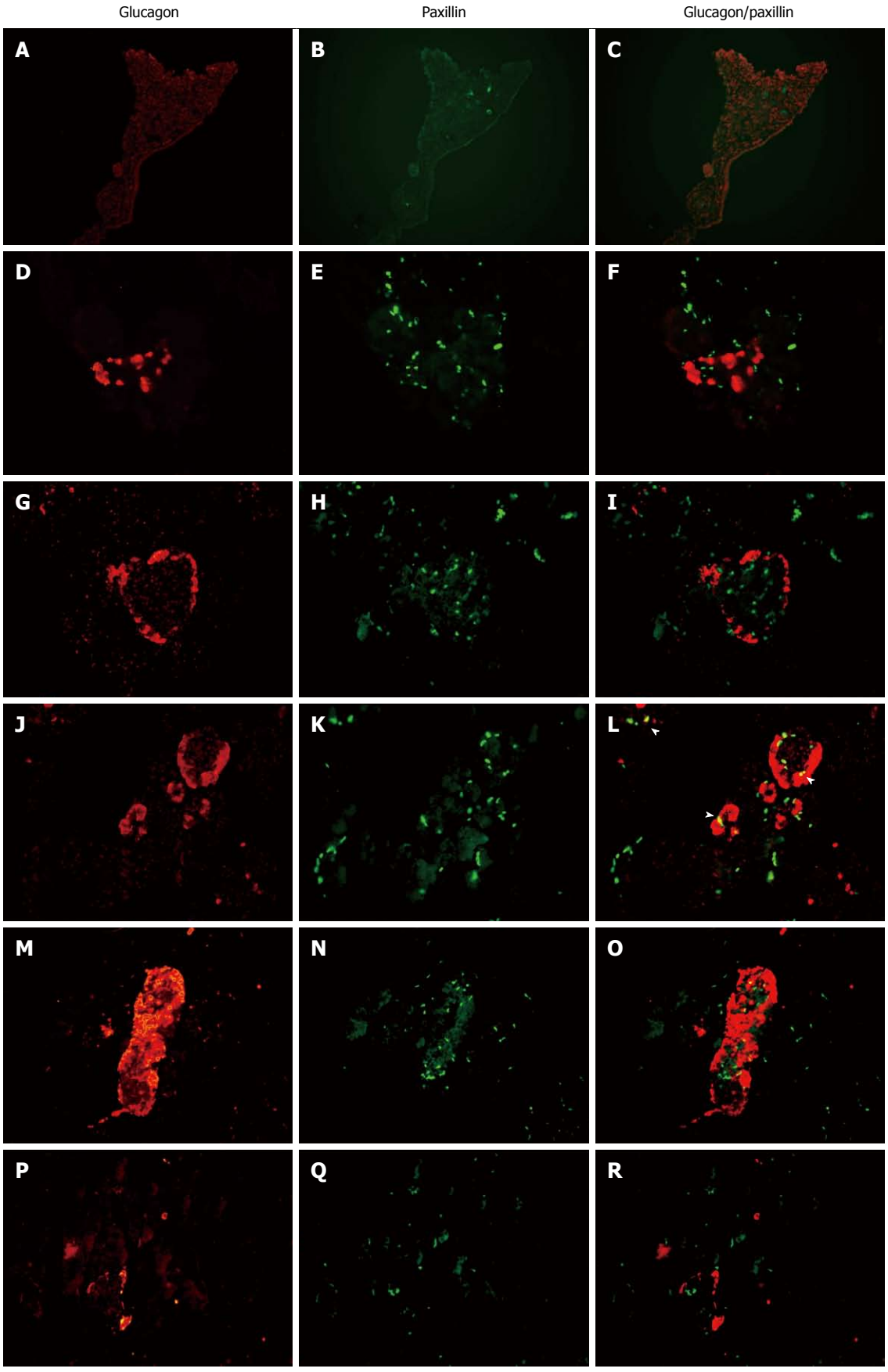

Figure 4 Immunofluorescent localization of paxillin and glucagon in the pancreas of E15.5, E18.5, P0, P7, P21 and adult rats. The paxillin antibody was detected with an fluorescein isothiocyanate (green)-labeled secondary antibody and the glucagon antibody was detected with a Cy3 (red)-labeled secondary antibody. Overlap between paxillin (green) and glucagon (red) labeling is indicated by arrowheads. Original magnification 400 ×. A-C: E15.5; D-F: E18.5; G-I: P0; J-L: P7; M-O: P21; P-R: Adult.

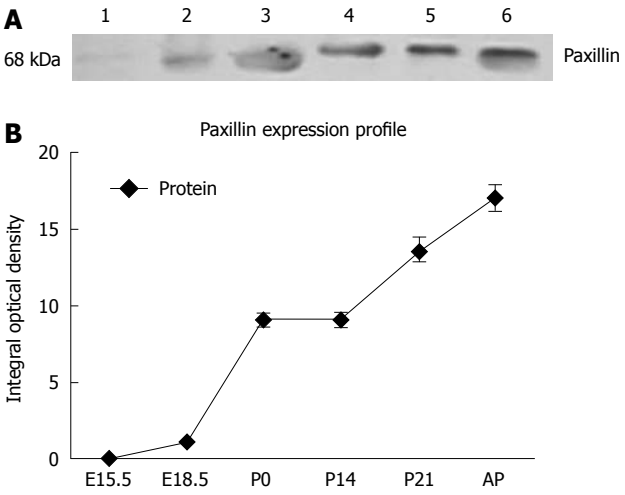

Figure 5 Paxillin protein expression level in the developing rat pancreas measured by Western blotting. A: From E15.5 to adulthood, paxillin protein (68 KD) expression in pancreas was highest at adult. 1: E15.5; 2: E18.5; 3: P0; 4: P14; 5: P21; 6: Adult; B: Progressively increased level of paxillin protein through the transition from E15.5 to adult is observed. Results are representative of three independent experiments.

forms of paxillin in the developmental process suggested their distinct physiological function[7].

DISCUSSION

During organogenesis, specialized cell types are generated from progenitor cell populations and are precisely organized into the elaborate structure of the adult organ[16,17]. Biochemical and ultrastructural studies delineated three distinct phases of pancreatic development in rats and mice. The first is during early organogenesis and is known as the "primary transitional phase" in which cytodifferentiation into exocrine or endocrine cells is minimal (approximately from E10 to E12/12.5 in rat). This is followed by the "proto-differentiated state" (from E12.5 to E15.5), in which epithelial cell proliferation is high, leading to rapid growth and lobulation, while cytodifferentiation is poor. During late organogenesis, a third phase or "secondary transitional phase" (from E15.5 to E18.5) is detected in which a dramatic increase in the number of endocrine and exocrine cells with high levels of insulin and exocrine enzymes is observed. During the secondary transition, endocrine cell migration and adhesion, and primary islet formation are key events. At approximately E16 the islet progenitor cells leave the contiguous epithelium, migrate through the adjacent ECM into the surrounding mesenchyme, and aggregate to form the islets of Langerhans. The islets are not fully formed until shortly before birth in E18-E19 and undergo further remodeling and maturation for 2-3 wk after birth[4]. Although some studies have addressed the function of single gene, such as integrins in islet formation, much less is known about the multifactor network that guides islet formation during embryogenesis[18-20]. The present study demonstrates that pancreatic development is accompanied by a specific spatiotemporal pattern of protein and mRNA expression of paxillin, which is a mediator in integrin signaling, suggesting a role for paxillin during pancreatic development.

The protein and mRNA expression of paxillin in the whole pancreas determines a specific temporal pattern of expression from embryonic development to postnatal development. High mRNA expression, along with low protein level of paxillin, was observed in the embryonic pancreas (Figures 1 and 5). Subsequently, into postnatal life, decreased paxillin mRNA expression was paralleled to increased protein levels in postnatal life. What is more, genes related to paxillin, such as ILK, clathrin, vinculin and integrin β1, shared the same expression pattern with paxillin. The highest expression level of these genes was almost detected at E15.5 or E18.5. In the present study, a dramatic increase of paxillin protein expression in the neonatal pancreas, compared with embryonic period, was detected. From the 18th day of gestation until birth, active cellular reorganization takes place to form the classic cellular features of the islet. After birth, apoptosis of a great amount of islet cells take place. From P14 to P21, islet cell proliferation increased dramatically and islet structure is remodeled. Cell adhesion and migration are necessary to islet structure remodeling. Immunohistochemical staining of paxillin demonstrated its expression within rat islets at P14 and P21. To the best of our knowledge, there have been no reports concerning the expression of paxillin in islets of Langerhans. Given the pivotal role of paxillin in cell adhesion and migration, the specific high expression of paxillin protein after birth indicates a potential role in islet structure remodeling.

The expression of paxillin at both the mRNA and protein level indicates that paxillin may be critical in mediating the biological functions of the developing pancreas, especially in maintaining islet structure and function. Regardless, these studies demonstrate that paxillin is expressed at the protein and mRNA level throughout development, indicating potential molecular signals mediated by paxillin that may control various aspects of pancreatic function and development.

In higher eukaryotes, paxillin exists as multiple isoforms (α, β, γ and δ)[6]. Paxillin α is the principal, ubiquitously expressed isoform, whereas the β- and γ-isoforms exhibit restricted expression. The β- and γ-isoforms contain a 34- and 48-amino acid insertion, respectively, between amino acids 277/278. However, there have been reports suggesting the lack of a murine γ-isoform[21,22]. Our study revealed that there exist two isoforms at 68 KD of paxillin protein in the pancreas during development, with different expression profiles. During the embryonic and neonatal development phases, only the smaller isoform was detected, while the larger isoform was detected only after birth. Interestingly, in the adult pancreas, the two isoforms could both be identified. It has been documented in a previous study that different paxillin isoforms show a distinct expression profile in whole embryos during development in mice, which suggested distinct physiological roles for each isoform[7]. Thus, the differential expression of paxillin isoforms in the pancreas during development may indicate their distinct roles in pancreas

development. Of course, further studies are needed to verify our hypothesis.

The morphogenesis of embryos depends both on interactions between cells and their surrounding ECM *via* integrin complexes, and direct cell-cell borders perceived as discrete adhesion systems that may interact with each other during morphogenesis[5,23-26]. The integrin family of transmembrane receptors physically connects the actin cytoskeleton of the cells to the ECM at focal adhesions, and thus, mediates migration and adhesion in many cells. The importance of focal adhesion in early development is well illustrated by mouse embryos deficient in fibronectin or focal adhesion components, including focal adhesion kinase (FAK), paxillin, or integrins. These embryos die early in development (days 5-10) with mesodermal defects. Data from previous studies support roles for paxillin and FAK in the organization of actin around tight junctions or adherens junctions; functions that would help stabilize or remodel cell-cell borders during the migration of this epithelial sheet[9,12].

In summary, our present study has, for the first time, demonstrated that paxillin is localized to the islets of Langerhans in developing rat pancreata *via* immunohistochemistry and immunofluorescence. Given the important roles of paxillin in intercellular adhesion and cell migration, it may play important roles in pancreas islet formation, which is a process of cell-cell adhesion and cell migration, especially in islet structure and functional maintenance. Further investigations are needed to verify our hypothesis.

ACKNOWLEDGMENTS

Special thanks to Dr. Ying-Bing Ge for his technical skills. Our gratitude also goes to Dr. Zheng-Xian Tao for his comments and criticisms.

COMMENTS

Background

Knowledge of the regional and temporal expression of paxillin will be useful in understanding its potential role in pancreatic development. However, no study has investigated the relationship between paxillin expression and pancreas development, and the expression of paxillin during pancreatic development in rats is poorly understood.

Research frontiers

The authors examined the expression of paxillin in rat pancreas during development.

Innovations and breakthroughs

The study, for the first time, demonstrated that paxillin is localized to the islets of Langerhans in developing rat pancreata *via* immunohistochemistry and immunofluorescence. Given the important roles of paxillin in intercellular adhesion and cell migration, it may play important roles in pancreas islet formation, which is a process of cell-cell adhesion and cell migration, especially in islet structure and functional maintenance.

Peer review

This is an interesting and fairly well performed study.

REFERENCES

1 **Gittes GK**. Developmental biology of the pancreas: a comprehensive review. *Dev Biol* 2009; **326**: 4-35
2 **Kim SK**, Hebrok M. Intercellular signals regulating pancreas development and function. *Genes Dev* 2001; **15**: 111-127
3 **Osman NM**, Kagohashi Y, Udagawa J, Otani H. Alpha1,4-N-acetylglucosaminyltransferase encoding gene EXTL3 expression pattern in mouse adult and developing tissues with special attention to the pancreas. *Anat Embryol* (Berl) 2003; **207**: 333-341
4 **Habener JF**, Kemp DM, Thomas MK. Minireview: transcriptional regulation in pancreatic development. *Endocrinology* 2005; **146**: 1025-1034
5 **Yashpal NK**, Li J, Wheeler MB, Wang R. Expression of {beta}1 integrin receptors during rat pancreas development--sites and dynamics. *Endocrinology* 2005; **146**: 1798-1807
6 **Brown MC**, Turner CE. Paxillin: adapting to change. *Physiol Rev* 2004; **84**: 1315-1339
7 **Mazaki Y**, Uchida H, Hino O, Hashimoto S, Sabe H. Paxillin isoforms in mouse. Lack of the gamma isoform and developmentally specific beta isoform expression. *J Biol Chem* 1998; **273**: 22435-22441
8 **Mazaki Y**, Hashimoto S, Sabe H. Monocyte cells and cancer cells express novel paxillin isoforms with different binding properties to focal adhesion proteins. *J Biol Chem* 1997; **272**: 7437-7444
9 **Crawford BD**, Henry CA, Clason TA, Becker AL, Hille MB. Activity and distribution of paxillin, focal adhesion kinase, and cadherin indicate cooperative roles during zebrafish morphogenesis. *Mol Biol Cell* 2003; **14**: 3065-3081
10 **Sorenson CM**, Sheibani N. Focal adhesion kinase, paxillin, and bcl-2: analysis of expression, phosphorylation, and association during morphogenesis. *Dev Dyn* 1999; **215**: 371-382
11 **Turner CE**. Paxillin and focal adhesion signalling. *Nat Cell Biol* 2000; **2**: E231-E236
12 **Schaller MD**. FAK and paxillin: regulators of N-cadherin adhesion and inhibitors of cell migration? *J Cell Biol* 2004; **166**: 157-159
13 **Hagel M**, George EL, Kim A, Tamimi R, Opitz SL, Turner CE, Imamoto A, Thomas SM. The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. *Mol Cell Biol* 2002; **22**: 901-915
14 **Shi J**, Ni XF, Chen Y. Patterning biomolecules with a water-soluble release and protection interlayer. *Langmuir* 2007; **23**: 11377-11380
15 **Andreolotti AG**, Bragado MJ, Tapia JA, Jensen RT, Garcia-Marin LJ. Cholecystokinin rapidly stimulates CrkII function in vivo in rat pancreatic acini. Formation of CrkII-protein complexes. *Eur J Biochem* 2003; **270**: 4706-4713
16 **Peters J**, Jürgensen A, Klöppel G. Ontogeny, differentiation and growth of the endocrine pancreas. *Virchows Arch* 2000; **436**: 527-538
17 **Piper K**, Brickwood S, Turnpenny LW, Cameron IT, Ball SG, Wilson DI, Hanley NA. Beta cell differentiation during early human pancreas development. *J Endocrinol* 2004; **181**: 11-23
18 **Mfopou JK**, Willems E, Leyns L, Bouwens L. Expression of regulatory genes for pancreas development during murine embryonic stem cell differentiation. *Int J Dev Biol* 2005; **49**: 915-922
19 **Gu G**, Wells JM, Dombkowski D, Preffer F, Aronow B, Melton DA. Global expression analysis of gene regulatory pathways during endocrine pancreatic development. *Development* 2004; **131**: 165-179
20 **Min BH**, Jeong SY, Kang SW, Crabo BG, Foster DN, Chun BG, Bendayan M, Park IS. Transient expression of clusterin (sulfated glycoprotein-2) during development of rat pancreas. *J Endocrinol* 1998; **158**: 43-52
21 **Bukharova T**, Weijer G, Bosgraaf L, Dormann D, van Haastert PJ, Weijer CJ. Paxillin is required for cell-substrate adhesion, cell sorting and slug migration during Dictyostelium development. *J Cell Sci* 2005; **118**: 4295-4310
22 **Tumbarello DA**, Brown MC, Hetey SE, Turner CE. Regula-

tion of paxillin family members during epithelial-mesenchymal transformation: a putative role for paxillin delta. *J Cell Sci* 2005; **118**: 4849-4863

23 **Bosco D**, Meda P, Halban PA, Rouiller DG. Importance of cell-matrix interactions in rat islet beta-cell secretion in vitro: role of alpha6beta1 integrin. *Diabetes* 2000; **49**: 233-243

24 **Cirulli V**, Beattie GM, Klier G, Ellisman M, Ricordi C, Quaranta V, Frasier F, Ishii JK, Hayek A, Salomon DR. Expression and function of alpha(v)beta(3) and alpha(v)beta(5) integrins in the developing pancreas: roles in the adhesion and migration of putative endocrine progenitor cells. *J Cell Biol* 2000; **150**: 1445-1460

25 **Bertelli E**, Bendayan M. Association between endocrine pancreas and ductal system. More than an epiphenomenon of endocrine differentiation and development? *J Histochem Cytochem* 2005; **53**: 1071-1086

26 **Wang R**, Li J, Lyte K, Yashpal NK, Fellows F, Goodyer CG. Role for beta1 integrin and its associated alpha3, alpha5, and alpha6 subunits in development of the human fetal pancreas. *Diabetes* 2005; **54**: 2080-2089

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BRIEF ARTICLE

αv integrin: A new gastrin target in human pancreatic cancer cells

Celine Cayrol, Claudine Bertrand, Aline Kowalski-Chauvel, Laurence Daulhac, Elizabeth Cohen-Jonathan-Moyal, Audrey Ferrand, Catherine Seva

Celine Cayrol, Claudine Bertrand, Aline Kowalski-Chauvel, Elizabeth Cohen-Jonathan-Moyal, Audrey Ferrand, Catherine Seva, INSERM UMR1037-Cancer Research Center of Toulouse, Paul Sabatier University Toulouse Ⅲ, 31432 Toulouse cedex 4, France
Laurence Daulhac, INSERM U766, Department of pharmacology, Clermont 1 University of Pharmacy, 63001 Clermont-Ferrand, France
Author contributions: Ferrand A and Seva C planned the study and wrote the paper; Daulhac L and Cohen-Jonathan-Moyal E contributed to the design of the study and critically revised the manuscript; Cayrol C, Bertrand C and Kowalski-Chauvel A were responsible for data acquisition and analysis.
Supported by Grants from INSERM
Correspondence to: Dr. Catherine Seva, INSERM UMR1037-Cancer Research Center of Toulouse, Paul Sabatier University Toulouse Ⅲ, 1 avenue J Poulhes, BP 84225, 31432 Toulouse cedex 4, France. cathy.seva@inserm.fr
Telephone: +33-5-61322408 Fax: +33-5-61322403
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Abstract

AIM: To analyse αv integrin expression induced by gastrin in pancreatic cancer models.

METHODS: αv integrin mRNA expression in human pancreatic cancer cells was analysed using a "cancer genes" array and confirmed by real-time reverse transcription-polymerase chain reaction (PCR). Western blotting and semi-quantitative immunohistochemistry were used to examine protein levels in human pancreatic cancer cell lines and pancreatic tissues, respectively. The role of αv integrin on gastrin-induced cell adhesion was examined using blocking anti-αv integrin monoclonal antibodies. Adherent cells were quantified by staining with crystal violet.

RESULTS: Using a "cancer genes" array we identified αv integrin as a new gastrin target gene in human pancreatic cancer cells. A quantitative real-time PCR approach was used to confirm αv integrin gene expression. We also demonstrate that Src family kinases and the PI 3-kinase, two signalling pathways specifically activated by the CCK-2 receptor (CCK2R), are involved in gastrin-mediated αv integrin expression. In contrast, inhibition of the ERK pathway was without any effect on αv integrin expression induced by gastrin. Our results also show that gastrin modulates cell adhesion *via* αv integrins. Indeed, *in vitro* adhesion assays performed on fibronectin show that gastrin significantly increases adhesion of pancreatic cancer cells. The use of blocking anti-αv integrin monoclonal antibodies completely reversed the increase in cell-substrate adhesion induced by gastrin. In addition, we showed *in vivo* that the targeted CCK2R expression in the pancreas of Elas-CCK2 mice, leads to the overexpression of αv integrin. This process may contribute to pancreatic tumour development observed in these transgenic animals.

CONCLUSION: αv integrin is a new gastrin target in pancreatic cancer models and contributes to gastrin effects on cell adhesion.

Key words: αv integrin; Cell adhesion; CCK-2 receptor; Gastrin; Pancreatic cancer

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INTRODUCTION

Pancreatic cancer has a poor prognosis with a 5-year survival rate < 5%. Despite intensive efforts to improve therapy, treatment remains unsatisfactory and most patients die within months as a result of rapid local spread of tumour or metastatic dissemination[1]. This poor prognosis is mainly due to the propensity of this tumour to invade the adjacent structures and metastasize to distant organs early in the course of this disease; however, the molecular basis for these characteristics of pancreatic cancer is incompletely understood. A better understanding of the genes involved in tumour growth and migration may allow development of novel treatment strategies to rapidly tackle this disease.

Several lines of evidence support the role of gastrin, a digestive peptide hormone and its G protein-coupled receptor (CCK2R) in pancreatic cancer development. Gastrin and its receptor are up-regulated in human pancreatic adenocarcinoma as well as in preneoplastic lesions[2,3]. A splice variant of the CCK2R has recently been identified, which has constitutive activity and is exclusively expressed in certain human colon and pancreatic cancers[4-6]. In addition, we have reported in Elas-CCK2 transgenic mice, expressing functional human CCK2R in pancreatic exocrine cells, an increased pancreatic growth, an acinar to ductal trans-differentiation, postulated to be a preneoplastic step in pancreatic carcinogenesis and the development of tumours[7,8].

Besides proliferation, gastrin has been shown to modulate cell adhesion and migration. We and others have recently demonstrated *in vitro* that prolonged activation of the CCK2R by gastrin induces stress fibre formation, alters cell morphology, increases loss of cell-cell adhesion, as well as motility of epithelial cells[9-12]. We have also shown the loss of intercellular adhesion in acini of Elas-CCK2 mice before tumour formation[13].

Several signalling pathways activated by the CCK2R have been implicated in the proliferative effects or cell migration induced by gastrin. They include: MAP-kinases[14,15], the phosphatidylinositol 3-kinase and the JAK2/STAT3 pathway[16,17]. In addition, Src family tyrosine kinases and p125FAK have also been shown to play a crucial role in these biological effects of gastrin[18].

In gastric epithelial cells, several target genes of the CCK2R have already been identified. They include genes involved in gastric acid secretion[19], early response genes, c-Fos[20], c-Jun and c-Myc[21,22] and other growth-related genes such as cyclin D1[23], Reg-1[24], or the HB-EGF[25]. In addition, in the same cellular model, gastrin also regulates the expression of genes associated with cell migration and invasion such as the *MMP9* gene, a matrix metalloproteinase[26]. In several cellular models such as gastric and colonic cancer cells, intestinal epithelial cells or fibroblasts transfected with the CCK2R, gastrin has also been shown to enhance *cyclooxygenase-2* gene expression, known to play an important role in inflammation processes and carcinogenesis[27-29].

In contrast, to our knowledge, very few gastrin-regulated genes have been identified in pancreatic models expressing the CCK2R. Recently, we showed that Reg proteins are targets of CCK2R activation and are induced during the early steps of carcinogenesis in Elas-CCK2 mouse pancreas[30]. In addition, we also identified β_1 integrin as a gastrin-regulated gene in human pancreatic cancer cells and demonstrated its involvement in modulation of cell adhesion by the CCK2R[31].

In this study, we identified αv integrin, another member of the large integrin family, as a new gastrin target in the human pancreatic cancer cell line, Panc-1. Integrins which mediate cell adhesion play an important role in cell migration, survival and differentiation. Here we show *in vitro* that αv integrin is involved in the modulation of cell adhesion by the CCK2R. In addition, we demonstrate *in vivo* that the targeted CCK2R expression in the pancreas of Elas-CCK2 mice, which present preneoplastic lesions and develop pancreatic tumours, leads to αv integrin expression.

MATERIALS AND METHODS

Cell culture

The human pancreatic cancer cell line, Panc-1 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, United States). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS at 37 ℃ in a humidified atmosphere containing 5% CO_2. In all experiments, cells were serum-starved for 18 h prior to gastrin stimulation. Human gastrin $_{2\text{-}17ds}$ (Bachem, Switzerland) was used in all experiments.

RNA extraction and reverse transcription

Total RNA was isolated from Panc-1 cells treated with or without gastrin as indicated using the RNeasy RNA Isolation Kit (Qiagen, Valencia, CA, United States). After pretreating RNA with 10 units DNase (Invitrogen, Carlsbad, CA, United States), cDNA was produced from 1 μg of total RNA using the Superscript First-Strand Synthesis System for reverse transcription-polymerase chain reaction (PCR) (Invitrogen, Carlsbad, CA, United States).

"Cancer super array"

A specific "Cancer array" (96 genes) from SuperArray (Bioscience Corporation, Beverly, MA, United States) was used in this study. Total RNA was isolated from Panc-1 cells as described above. Reverse transcription of cellular RNA was carried out with the RT-Labeling kit (SuperArray, Bioscience Corporation, Beverly, MA,

United States) according to the manufacturer's instructions. The biotinylated probes from gastrin-stimulated cells and unstimulated cells were hybridized overnight to separate membranes at 60 ℃, washed with SSC/SDS solutions, incubated with the avidin-alkaline phosphatase conjugate and exposed to a chemiluminescent substrate. Analysis of the images and quantitation of the spots in both membranes were performed by the ScanAlyze 2.5 software, and normalization of the values and comparison of the intensities was achieved by the GE ArrayAnalyzer 1.3 (SuperArray, Bioscience Corporation, Beverly, MA, United States) software.

Real-time PCR

αv integrin expression was determined *via* real-time PCR, using fluorescent SYBR green dye (Applied Biosystems, Framingham, MA, United States) to allow semi quantitative analysis of gene expression levels. Amplification was conducted using ABI-Stepone + Detection System (Applied Biosystems, Framingham, MA, United States). Relative fold changes were determined using the 2-ΔΔCT method, in which *18S* gene was used for normalization.

Primers used (18S: forward-CGCAGCTAGGAATAATGGAATAGG, reverse-CATGGCCTCAGTTCCGAAA; αv integrin: forward-TGCCCAGCGCGTCTTC, reverse-TGGGTGGTGTTTGCTTTGG).

Western blotting

Western blotting analyses were performed on lysates from Panc-1 cells stimulated or not with gastrin. Fractions, containing identical levels of proteins, were separated by SDS-PAGE and analyzed by Western blotting with the indicated antibodies. The immunoreactivity was visualized with an enhanced chemiluminescence system (Pierce, IL, United States). Anti-αv integrin antibodies were from Chemicon (Temecula, CA, United States).

Cell adhesion assay

Cell adhesion assays were carried out in 96-well plates using 10^5 cells/cm^2 in a final volume of 100 μL/well of serum-free medium. Wells were coated overnight at 4 ℃ with fibronectin diluted at 5 μg/mL in phosphate buffered solution (PBS) then washed twice with 100 μL of PBS and blocked with 1% bovine serum albumin (BSA)-PBS for 30 min at room temperature before addition of the cell suspension. The cells were incubated for 2 h at 37 ℃ with or without gastrin. Adherent cells were fixed with 50 μL of 96% ethanol for 10 min, stained with 50 μL of 0.1% crystal violet, rinsed extensively with water and dried at room temperature. Stained cells were solubilised with 50 μL of 0.2% Triton X-100 and quantified by measuring the absorbance at 570 nm. For adhesion inhibition experiments, cells were pretreated for 30 min at 37 ℃ with or without 5 μg/mL function-blocking antibodies directed against αv integrin and treated or not with gastrin for 2 h.

Animals

Homozygous Elas-CCK2 mice used in this study have been described previously[8]. Homozygous Elas-CCK2 mice in a B6SJLF1 background 3 at least 6-mo old and 3 corresponding control littermates were used. Mice were reared in a routine animal facility of the I2MR and maintained on a 12:12 h light-dark cycle. All the experiments were performed during the daytime. All procedures were approved by the I2MR Animal Facility Care Committee.

Immunohistochemistry

Mice were killed by decapitation, the pancreas was excised, fixed and embedded in paraffin using standard techniques. Immunohistochemistry was performed as previously described[16] using anti-αv integrin antibodies (Chemicon, Temecula, United States). Sections were incubated with the appropriate secondary and tertiary peroxidase-labelled antisera (DAKO, Glostrup, Denmark) at room temperature, exposed to a solution of diaminobenzidine. All dilutions and washes were performed with phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin.

Statistical analysis

All results are presented as mean ± SE. Statistical significance was calculated using unpaired Student's *t* test. Values of $P < 0.05$ were considered statistically significant. All analyses were performed using "GraphPad Prism" software.

RESULTS

Gastrin increases αv integrin expression in Panc-1 cells

In order to identify new gastrin-regulated genes, a human cancer array of 96 genes was probed with samples from either control Panc-1 cells or cells treated with gastrin for 24 h.

Among the genes positively modulated by the CCK2R in these experiments, we observed a significant increase in the expression of αv integrin (Figure 1A). A quantitative real-time PCR approach was used to confirm and quantify the αv integrin gene expression. In response to gastrin, the increase in αv integrin gene expression was time-dependent. A significant effect due to gastrin was detectable 3 h after treatment. At 24 h, we observed a 5-fold increase in the expression of αv integrin in response to gastrin (Figure 1B).

In addition, we also confirmed the increase in protein levels of αv integrin in gastrin-stimulated cells using Western blotting analysis (Figure 1C and D).

Signalling pathways involved in αv integrin expression stimulated by gastrin

As mentioned in the Introduction, gastrin exerts its trophic effects and modulates cell adhesion through a variety of intracellular pathways depending on the cellular model. We previously identified the signalling pathways specifically activated by the CCK2R in Panc-1 cells[31]. They include the ERK pathway, the PI3K/AKT pathway

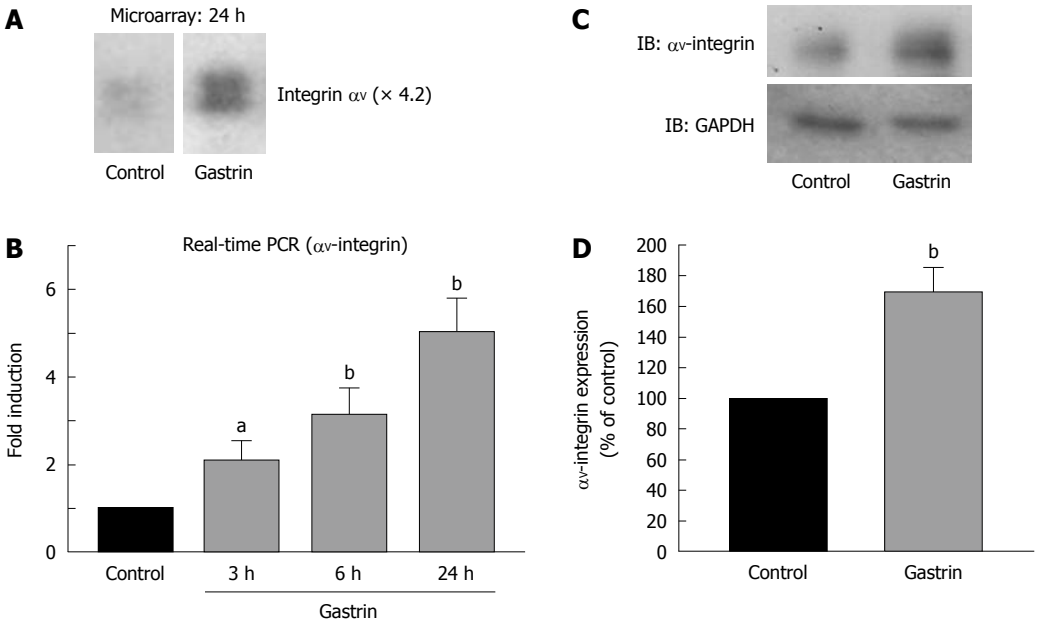

Figure 1 Increased expression of αv integrin in response to gastrin in Panc-1 cells. A: Results of probing a 96 genes array with samples from unstimulated Panc-1 cells (control) or cells stimulated with 100 nmol/L of gastrin for 48 h; B: Real time polymerase chain reaction (PCR) analysis of αv integrin mRNA expression in Panc-1 cells. Cells were treated or not with gastrin for the time indicated. Total RNA was isolated and αv integrin mRNA expression was determined by real time PCR as described Materials and Methods; C, D: Expression of αv integrin protein was examined by Western analysis following treatment of the cells with gastrin for 24 h. Blots were also probed with an antibody against GAPDH to ensure equal loading of proteins. Representative data from 3 experiments are shown. Quantifications of three experiments are presented as mean ± SE. Significance was accepted at $P \leq 0.05$, $^{a}P < 0.05$, $^{b}P < 0.01$.

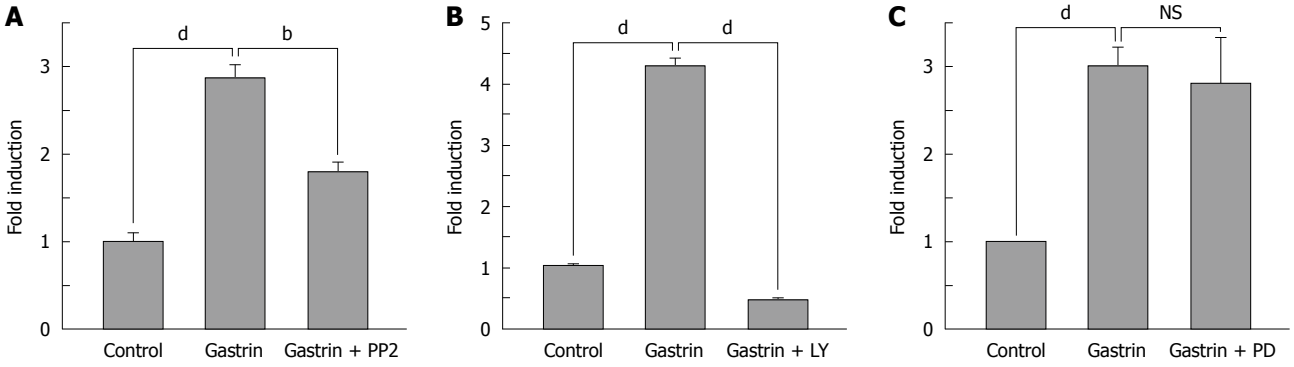

Figure 2 Signalling pathways involved in αv integrin expression stimulated by gastrin. Cells were pretreated for 30 min with (B) a specific Pi3K inhibitor (LY294002, 20 μmol/L), (A) a Src-kinase inhibitor (PP2, 30 μmol/L) or (C) a MEK inhibitor (PD PD098059, 20 μmol/L) prior to gastrin stimulation. After 24 h, total RNA was isolated. Quantitative real-time polymerase chain reaction was performed as described in Materials and Methods. Quantifications of three experiments are presented as mean ± SE. Significance was accepted at $P \leq 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$. NS: Not significant.

and the activation of Src-kinases.

To determine the cellular mechanism by which gastrin increased αv integrin gene expression, we examined gastrin-regulated αv integrin gene expression in Panc-1 by quantitative real-time PCR in the absence or presence of different specific inhibitors, LY294002, PP2, or PD098059 which block the PI 3-kinase pathway, Src family kinases and the ERK pathway, respectively. When cells were pre-incubated with PP2, the response to gastrin was decreased by 60% and totally blocked in cells pre-treated with LY294002 (Figure 2A and B), whereas the inhibitors alone did not significantly affect basal αv integrin expression (PP2: 1.09 ± 0.2 fold induction, LY204002: 0.93 ± 0.35 fold induction). These results indicate that Src family kinases and the PI 3-kinase pathway mediate gastrin-increased αv integrin gene expression in Panc-1 cells. In contrast, the inhibitor of the ERK pathway was without any effect (Figure 2C).

Effect of gastrin on Panc-1 cell adhesion to fibronectin

In this study, we have identified αv integrin as a new gastrin target in Panc-1 cells. Integrins act as adhesion receptors linking the extracellular matrix (ECM) to the cytoskeleton. For many cell types, integrin-mediated adhesion is required for cell growth and cell survival. In the second part of this study, we investigated whether gastrin had an effect on Panc-1 cell adhesion. In a cell adhesion assay using fibronectin-coated wells, we showed

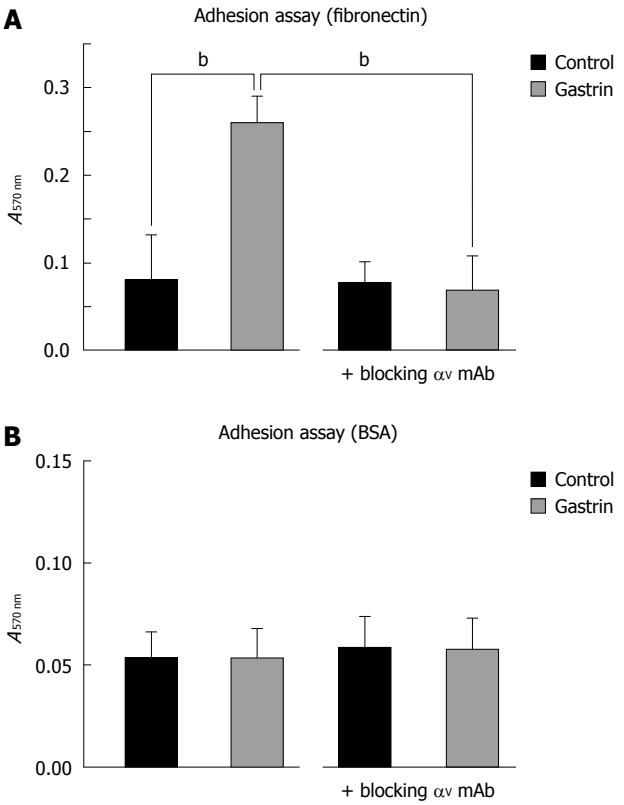

Figure 3 Effect of gastrin on Panc-1 cell adhesion. Cells were added to fibronectin-coated (A) or non-coated (BSA alone) (B) 96-wells for 2 h in the presence or absence of gastrin. Adherent cells were fixed and stained with crystal violet as described in Materials and Methods. After solubilisation, absorbance was measured at 570 nm. When indicated, Panc-1 cells were pre-incubated, with a blocking αv mAb for 30 min prior to gastrin treatment for 2 h. Quantifications of three experiments are presented as mean ± SE. Significance was accepted at $P \leqslant 0.05$. $^{b}P < 0.01$. BSA: Bovine serum albumin.

that gastrin induced a significant increase in Panc-1 cell adhesion (Figure 3A). As expected in BSA only controls, we did not observe any effect of gastrin on cell adhesion (Figure 3B).

To determine the role of αv integrin in gastrin-enhanced Panc-1 cell adhesion, we used blocking anti-αv integrin monoclonal antibodies. When added 30 min prior to gastrin stimulation, the antibodies significantly decreased gastrin-stimulated Panc-1 cell adhesion (Figure 3A). This confirmed that αv integrin plays an important role in Panc-1 cell adhesion stimulated by gastrin.

Immunohistochemical staining of αv integrin in the pancreas of Elas-CCK2 mice

We recently described that Elas-CCK2 mice express human CCK2R in acini. These mice exhibited an increased pancreatic growth, an acinar to ductal trans-differentiation, postulated to be a preneoplastic step in pancreatic carcinogenesis, and developed tumors[8].

Thus, to analyse *in vivo* the relevance of αv integrin expression in correlation to CCK2R expression, we analysed αv integrin overexpression in pancreatic tissue sections from Elas-CCK2 mice and control littermates using immunohistochemistry methods. As shown in Figure 4, tissues derived from Elas-CCK2 mice showed an upregulation of αv integrin (Figure 4B) as compared to control mice (Figure 4A).

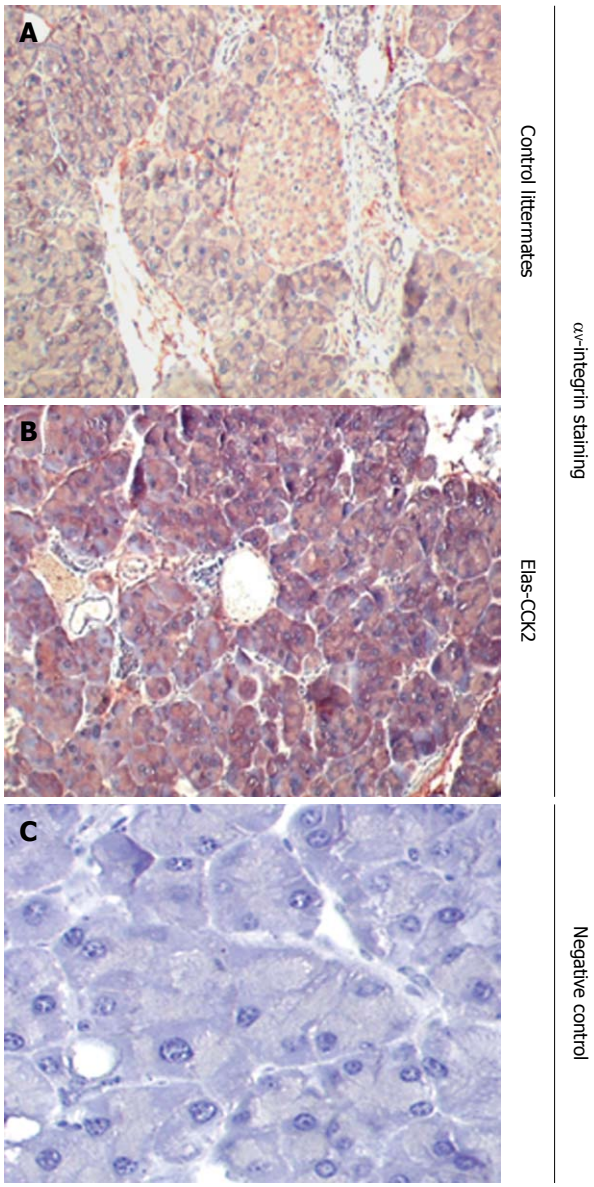

Figure 4 Overexpression of αv integrin in the pancreas of Elas-CCK2 mice. Immunohistochemistry analysis of paraffin-embedded pancreatic tissues from Elas-CCK2 mice and control littermates were performed using antibodies specific for αv integrin (A, B). Representative data from 3 experiments (3 different animals in each group) are shown. A negative control without secondary antibody was also included (C).

DISCUSSION

Several lines of evidence suggest that gastrin and CCK2R could contribute to pancreatic carcinogenesis by modulating processes such as proliferation, cell adhesion or migration. In the current study, we identified αv integrin as a new gastrin-regulated gene in human pancreatic cancer cells and demonstrated its involvement in modulation of

cell adhesion by gastrin.

Integrins, a large family of cell-surface receptors, act as the bridge between ECM proteins and cytoskeletal proteins[32]. They are crucial for cell migration but also modulate signal transduction cascades implicated in cell survival or proliferation. Several studies have demonstrated that integrins played a key role in the malignant behaviour of neoplastic cells and were important mediators of tumour invasion and metastasis formation through interactions with ECM proteins[33-35]. Alterations in integrin expression have been correlated with aggressive growth and metastatic capacity of several tumours[36-40]. In addition, several integrin subunits are upregulated in pancreatic carcinoma, in particular the fibronectin receptor β_1 and β_3 integrins, two subunits known to interact with α_v integrin[41-43]. We previously identified β_1 integrin as a gastrin target in pancreatic cancer[31]. Here, we show that gastrin increases the expression of another member of the integrin family, α_v integrin, at the mRNA and protein level in a human pancreatic tumour cell line. In addition, the use of blocking anti-α_v integrin monoclonal antibodies completely reversed the increase in cell-substrate adhesion induced by gastrin. Previously we showed an inhibitory effect of anti-β_1 integrin antibodies on gastrin-induced cell adhesion, suggesting that the heterodimer $\alpha_v\beta_1$ might be important in gastrin signalling. However, since the β_3 subunit is also overexpressed in pancreatic adenocarcinomas and can interact with α_v subunit, it might be important to analyse, using anti-β_3 integrin, whether it also contributes to gastrin-induced cell adhesion.

In gastric cells, the regulation by gastrin of numerous genes, including genes involved in gastric acid secretion[19], early response genes[20] or genes associated with cell migration[26], involves the activation of the ERK1/2 pathway. In other cellular models such as colon cancer cells, the PI-3-kinase pathway is also involved in the regulation of gastrin target genes. To our knowledge, very little is known about gene regulation by gastrin in pancreatic tumour models. In this study, we demonstrated in pancreatic cancer cells that Src family kinases and the PI-3-kinase pathway play a crucial role in the expression of α_v integrin modulated by gastrin.

The present study and previously published studies by our group demonstrate that gastrin affects cell adhesion and migration by different complementary mechanisms. First, gastrin modulates cell-cell adhesion by inducing a dissociation of the E-cadherin-catenin-complex leading to cytoskeleton reorganization and cell invasion. Here, we show that gastrin also modulates cell-substrate adhesion *via* the α_v integrin.

Another important finding of this study is that the expression of a G protein-coupled receptor, namely the CCK2R, targeted in mouse pancreatic acinar tissue, leads to the over-expression of α_v integrin. These transgenic mice display an increased growth of the pancreas and develop preneoplastic lesions then pancreatic tumours presenting a ductal phenotype similar to that observed in human pancreatic tumours.

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We thank Dr. André F (Marseille, France) for blocking anti-α_v integrin antibodies. We thank Dr. Dufresne M (Toulouse, France) for providing us with pancreatic tissue sections from Elas-CCK2 mice and control mice.

COMMENTS

Background

Pancreatic cancer has a poor prognosis with a 5-year survival rate < 5%. Despite intensive efforts to improve therapy, treatment remains unsatisfactory and most patients die within months as a result of rapid local spread of tumour or metastatic dissemination. A better understanding of the genes involved in tumour growth and migration may allow the development of novel treatment strategies to rapidly tackle this disease.

Research frontiers

Integrins play a key role in the malignant behaviour of neoplastic cells and are important mediators of tumour growth invasion and metastasis. Several publications support the role of gastrin, a peptide hormone, in pancreatic cancer development. However, the mechanism by which gastrin regulates integrin signalling in pancreatic cancer has not been addressed. In this study, the authors show that regulation of α_v integrin by gastrin may contribute to pancreatic tumour development.

Innovations and breakthroughs

This is the first study to report that α_v integrin is a gastrin target in human pancreatic cancer cells. Furthermore, we identified the signalling pathways involved in gastrin-mediated α_v integrin expression. Another important finding of this study is that the expression of a G protein-coupled receptor, namely the CCK2R, targeted in mouse pancreatic acinar tissue, leads to the over-expression of α_v integrin. These transgenic mice display an increased growth of the pancreas and develop preneoplastic lesions then pancreatic tumours presenting a ductal phenotype similar to that observed in human pancreatic tumours.

Applications

A better understanding of the genes involved in tumour growth and migration may allow the development of novel treatment strategies for patients with pancreatic cancer.

Terminology

Integrins, a large family of cell-surface receptors, act as the bridge between extracellular matrix proteins and cytoskeletal proteins. They are crucial for cell migration but also modulate signal transduction cascades implicated in cell survival or proliferation.

Peer review

This is a very well written and clearly laid out manuscript. The authors appear to have carried out the experiments to a high standard and the data are convincing. There are one or two experimental controls that are not included however if the authors can include these or comment on the fact that their inclusion would strengthen their observations.

REFERENCES

1 **Chua YJ**, Cunningham D. Adjuvant treatment for resectable pancreatic cancer. *J Clin Oncol* 2005; **23**: 4532-4537
2 **Caplin M**, Savage K, Khan K, Brett B, Rode J, Varro A, Dhillon A. Expression and processing of gastrin in pancreatic adenocarcinoma. *Br J Surg* 2000; **87**: 1035-1040
3 **Goetze JP**, Nielsen FC, Burcharth F, Rehfeld JF. Closing the gastrin loop in pancreatic carcinoma: coexpression of gastrin and its receptor in solid human pancreatic adenocarcinoma. *Cancer* 2000; **88**: 2487-2494
4 **Hellmich MR**, Rui XL, Hellmich HL, Fleming RY, Evers BM, Townsend CM. Human colorectal cancers express a constitutively active cholecystokinin-B/gastrin receptor that stimulates cell growth. *J Biol Chem* 2000; **275**: 32122-32128

5 **Ding WQ**, Kuntz SM, Miller LJ. A misspliced form of the cholecystokinin-B/gastrin receptor in pancreatic carcinoma: role of reduced sellular U2AF35 and a suboptimal 3'-splicing site leading to retention of the fourth intron. *Cancer Res* 2002; **62**: 947-952
6 **Schmitz F**, Otte JM, Stechele HU, Reimann B, Banasiewicz T, Fölsch UR, Schmidt WE, Herzig KH. CCK-B/gastrin receptors in human colorectal cancer. *Eur J Clin Invest* 2001; **31**: 812-820
7 **Clerc P**, Saillan-Barreau C, Desbois C, Pradayrol L, Fourmy D, Dufresne M. Transgenic mice expressing cholecystokinin 2 receptors in the pancreas. *Pharmacol Toxicol* 2002; **91**: 321-326
8 **Clerc P**, Leung-Theung-Long S, Wang TC, Dockray GJ, Bouisson M, Delisle MB, Vaysse N, Pradayrol L, Fourmy D, Dufresne M. Expression of CCK2 receptors in the murine pancreas: proliferation, transdifferentiation of acinar cells, and neoplasia. *Gastroenterology* 2002; **122**: 428-437
9 **Bierkamp C**, Kowalski-Chauvel A, Dehez S, Fourmy D, Pradayrol L, Seva C. Gastrin mediated cholecystokinin-2 receptor activation induces loss of cell adhesion and scattering in epithelial MDCK cells. *Oncogene* 2002; **21**: 7656-7670
10 **Ferrand A**, Kowalski-Chauvel A, Bertrand C, Pradayrol L, Fourmy D, Dufresne M, Seva C. Involvement of JAK2 upstream of the PI 3-kinase in cell-cell adhesion regulation by gastrin. *Exp Cell Res* 2004; **301**: 128-138
11 **Noble F**, Roques BP. Phenotypes of mice with invalidation of cholecystokinin [CCK(1) or CCK(2)] receptors. *Neuropeptides* 2002; **36**: 157-170
12 **Taniguchi T**, Takaishi K, Murayama T, Ito M, Iwata N, Chihara K, Sasaki T, Takai Y, Matsui T. Cholecystokinin-B/gastrin receptors mediate rapid formation of actin stress fibers. *Oncogene* 1996; **12**: 1357-1360
13 **Bierkamp C**, Bonhoure S, Mathieu A, Clerc P, Fourmy D, Pradayrol L, Seva C, Dufresne M. Expression of cholecystokinin-2/gastrin receptor in the murine pancreas modulates cell adhesion and cell differentiation in vivo. *Am J Pathol* 2004; **165**: 2135-2145
14 **Daulhac L**, Kowalski-Chauvel A, Pradayrol L, Vaysse N, Seva C. Src-family tyrosine kinases in activation of ERK-1 and p85/p110-phosphatidylinositol 3-kinase by G/CCKB receptors. *J Biol Chem* 1999; **274**: 20657-20663
15 **Todisco A**, Takeuchi Y, Urumov A, Yamada J, Stepan VM, Yamada T. Molecular mechanisms for the growth factor action of gastrin. *Am J Physiol* 1997; **273**: G891-G898
16 **Ferrand A**, Kowalski-Chauvel A, Bertrand C, Escrieut C, Mathieu A, Portolan G, Pradayrol L, Fourmy D, Dufresne M, Seva C. A novel mechanism for JAK2 activation by a G protein-coupled receptor, the CCK2R: implication of this signaling pathway in pancreatic tumor models. *J Biol Chem* 2005; **280**: 10710-10715
17 **Kowalski-Chauvel A**, Pradayrol L, Vaysse N, Seva C. Gastrin stimulates tyrosine phosphorylation of insulin receptor substrate 1 and its association with Grb2 and the phosphatidylinositol 3-kinase. *J Biol Chem* 1996; **271**: 26356-26361
18 **Daulhac L**, Kowalski-Chauvel A, Pradayrol L, Vaysse N, Seva C. Gastrin stimulates the formation of a p60Src/p125FAK complex upstream of the phosphatidylinositol 3-kinase signaling pathway. *FEBS Lett* 1999; **445**: 251-255
19 **Höcker M**, Zhang Z, Merchant JL, Wang TC. Gastrin regulates the human histidine decarboxylase promoter through an AP-1-dependent mechanism. *Am J Physiol* 1997; **272**: G822-G830
20 **Stepan VM**, Tatewaki M, Matsushima M, Dickinson CJ, del Valle J, Todisco A. Gastrin induces c-fos gene transcription via multiple signaling pathways. *Am J Physiol* 1999; **276**: G415-G424
21 **Taniguchi T**, Matsui T, Ito M, Murayama T, Tsukamoto T, Katakami Y, Chiba T, Chihara K. Cholecystokinin-B/gastrin receptor signaling pathway involves tyrosine phosphorylations of p125FAK and p42MAP. *Oncogene* 1994; **9**: 861-867
22 **Wang JY**, Wang H, Johnson LR. Gastrin stimulates expression of protooncogene c-myc through a process involving polyamines in IEC-6 cells. *Am J Physiol* 1995; **269**: C1474-C1481
23 **Zhukova E**, Sinnett-Smith J, Wong H, Chiu T, Rozengurt E. CCK(B)/gastrin receptor mediates synergistic stimulation of DNA synthesis and cyclin D1, D3, and E expression in Swiss 3T3 cells. *J Cell Physiol* 2001; **189**: 291-305
24 **Fukui H**, Kinoshita Y, Maekawa T, Okada A, Waki S, Hassan S, Okamoto H, Chiba T. Regenerating gene protein may mediate gastric mucosal proliferation induced by hypergastrinemia in rats. *Gastroenterology* 1998; **115**: 1483-1493
25 **Sinclair NF**, Ai W, Raychowdhury R, Bi M, Wang TC, Koh TJ, McLaughlin JT. Gastrin regulates the heparin-binding epidermal-like growth factor promoter via a PKC/EGFR-dependent mechanism. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G992-G999
26 **Wroblewski LE**, Pritchard DM, Carter S, Varro A. Gastrin-stimulated gastric epithelial cell invasion: the role and mechanism of increased matrix metalloproteinase 9 expression. *Biochem J* 2002; **365**: 873-879
27 **Colucci R**, Blandizzi C, Tanini M, Vassalle C, Breschi MC, Del Tacca M. Gastrin promotes human colon cancer cell growth via CCK-2 receptor-mediated cyclooxygenase-2 induction and prostaglandin E2 production. *Br J Pharmacol* 2005; **144**: 338-348
28 **Guo YS**, Cheng JZ, Jin GF, Gutkind JS, Hellmich MR, Townsend CM. Gastrin stimulates cyclooxygenase-2 expression in intestinal epithelial cells through multiple signaling pathways. Evidence for involvement of ERK5 kinase and transactivation of the epidermal growth factor receptor. *J Biol Chem* 2002; **277**: 48755-48763
29 **Slice LW**, Hodikian R, Zhukova E. Gastrin and EGF synergistically induce cyclooxygenase-2 expression in Swiss 3T3 fibroblasts that express the CCK2 receptor. *J Cell Physiol* 2003; **196**: 454-463
30 **Gigoux V**, Clerc P, Sanchez D, Coll MG, Corominola H, Leung-Theung-Long S, Pénicaud L, Gomis R, Seva C, Fourmy D, Dufresne M. Reg genes are CCK2 receptor targets in ElasCCK2 mice pancreas. *Regul Pept* 2008; **146**: 88-98
31 **Cayrol C**, Clerc P, Bertrand C, Gigoux V, Portolan G, Fourmy D, Dufresne M, Seva C. Cholecystokinin-2 receptor modulates cell adhesion through beta 1-integrin in human pancreatic cancer cells. *Oncogene* 2006; **25**: 4421-4428
32 **Hynes RO**. Integrins: bidirectional, allosteric signaling machines. *Cell* 2002; **110**: 673-687
33 **Giancotti FG**, Ruoslahti E. Integrin signaling. *Science* 1999; **285**: 1028-1032
34 **Guo W**, Giancotti FG. Integrin signalling during tumour progression. *Nat Rev Mol Cell Biol* 2004; **5**: 816-826
35 **van der Flier A**, Sonnenberg A. Function and interactions of integrins. *Cell Tissue Res* 2001; **305**: 285-298
36 **Vogelmann R**, Kreuser ED, Adler G, Lutz MP. Integrin alpha6beta1 role in metastatic behavior of human pancreatic carcinoma cells. *Int J Cancer* 1999; **80**: 791-795
37 **Sawai H**, Funahashi H, Yamamoto M, Okada Y, Hayakawa T, Tanaka M, Takeyama H, Manabe T. Interleukin-1alpha enhances integrin alpha(6)beta(1) expression and metastatic capability of human pancreatic cancer. *Oncology* 2003; **65**: 167-173
38 **Ahmed N**, Riley C, Oliva K, Rice G, Quinn M. Ascites induces modulation of alpha6beta1 integrin and urokinase plasminogen activator receptor expression and associated functions in ovarian carcinoma. *Br J Cancer* 2005; **92**: 1475-1485
39 **Mayoral R**, Fernández-Martínez A, Boscá L, Martín-Sanz P. Prostaglandin E2 promotes migration and adhesion in he-

patocellular carcinoma cells. *Carcinogenesis* 2005; **26**: 753-761
40 **Menendez JA**, Vellon L, Mehmi I, Teng PK, Griggs DW, Lupu R. A novel CYR61-triggered 'CYR61-alphavbeta3 integrin loop' regulates breast cancer cell survival and chemosensitivity through activation of ERK1/ERK2 MAPK signaling pathway. *Oncogene* 2005; **24**: 761-779
41 **Linder S**, Castaños-Velez E, von Rosen A, Biberfeld P. Immunohistochemical expression of extracellular matrix proteins and adhesion molecules in pancreatic carcinoma. *Hepatogastroenterology* 2001; **48**: 1321-1327
42 **Löhr M**, Trautmann B, Göttler M, Peters S, Zauner I, Maier A, Klöppel G, Liebe S, Kreuser ED. Expression and function of receptors for extracellular matrix proteins in human ductal adenocarcinomas of the pancreas. *Pancreas* 1996; **12**: 248-259
43 **Shimoyama S**, Gansauge F, Gansauge S, Oohara T, Beger HG. Altered expression of extracellular matrix molecules and their receptors in chronic pancreatitis and pancreatic adenocarcinoma in comparison with normal pancreas. *Int J Pancreatol* 1995; **18**: 227-234

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BRIEF ARTICLE

Cell proliferation of esophageal squamous epithelium in erosive and non-erosive reflux disease

Carlo Calabrese, Lorenzo Montanaro, Giuseppina Liguori, Elisa Brighenti, Mauela Vici, Paolo Gionchetti, Fernando Rizzello, Massimo Campieri, Massimo Derenzini, Davide Trerè

Carlo Calabrese, Giuseppina Liguori, Paolo Gionchetti, Fernando Rizzello, Massimo Campieri, Department of Clinical Medicine, University of Bologna, 40138 Bologna, Italy
Lorenzo Montanaro, Elisa Brighenti, Mauela Vici, Davide Trerè, Department of Experimental Pathology, University of Bologna, 40138 Bologna, Italy
Massimo Derenzini, Clinical Department Radiological and Histocytopathological Sciences, University of Bologna, 40138 Bologna, Italy
Author contributions: Calabrese C, Montanaro L and Trerè D designed the research; Calabrese C, Montanaro L, Liguori G, Brighenti E, Vici M and Trerè D performed the research; Calabrese C, Montanaro L, Trerè D, Derenzini M, Gionchetti P, Rizzello F, Campieri M analyzed the data; Calabrese C, Montanaro L and Trerè D wrote the paper.
Correspondence to: Carlo Calabrese, MD, PhD, Department of Clinical Medicine, University of Bologna, 40138 Bologna, Italy. carlo.calabrese2@unibo.it
Telephone: +39-0516-364191 Fax: +39-0516-364191
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Abstract

AIM: To elucidate cell proliferation in erosive reflux disease (ERD) and non-erosive reflux disease (NERD), we evaluated markers in squamous epithelial cells.

METHODS: Thirty-four consecutive patients with gastroesophageal-reflux-disease-related symptoms (21 NERD and 13 ERD) were evaluated for the enrolment into the study. All patients underwent 24-h pH monitoring, standard endoscopy, and biopsy for histological evaluation. The expression of cyclins D and A was evaluated by real-time reverse transcription polymerase chain reaction (RT-PCR) from isolated epithelial cells. In all samples, analysis of the isolated cell population revealed the presence of epithelial cells only.

RESULTS: Real-time RT-PCR showed that, in patients with ERD, the relative expression of cyclin D1 mRNA in esophageal epithelium was strongly decreased in comparison with NERD patients. The mean value of relative expression of cyclin D1 mRNA in NERD patients was 3.44 ± 1.9, whereas in ERD patients, it was 1.32 ± 0.87 (P = 0.011). Real-time RT-PCR showed that, in patients with ERD, relative expression of cyclin A mRNA in esophageal epithelium was decreased in comparison with that in NERD patients (2.31 ± 2.87 *vs* 0.66 ± 1.11). The mean bromodeoxyuridine labeling index in the NERD patients was 5.42% ± 1.68%, whereas in ERD patients, it was 4.3% ± 1.59%.

CONCLUSION: We confirmed reduced epithelial proliferation in ERD compared with NERD patients, and that individuals who develop ERD are characterized by weaker epithelial cell proliferation.

Key words: Esophageal cell proliferation; Erosive reflux disease; Non-erosive reflux disease; Gastroesophageal reflux disease; Cyclin A; Cyclin D; Bromodeoxyuridine

Peer reviewer: Wojciech Blonski, MD, PhD, University of Pennsylvania, GI Research-Ground Centrex, 3400 Spruce St, Philadelphia, PA 19104, United States

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INTRODUCTION

Most of the patients with gastroesophageal reflux disease

(GERD) fall into one of two categories: non-erosive reflux disease (NERD) or erosive reflux disease (ERD). The two main phenotypes of GERD appear to have different pathophysiological and clinical characteristics. NERD is the most common phenotypic presentation of GERD. Although separation of ERD and NERD on a clinical level is difficult, there are clearly physiological, pathophysiological, anatomical, and even histological characteristics that are unique to NERD. Natural course studies have demonstrated that most NERD patients do not progress over time to ERD or even Barrett's esophagus. NERD patients compared to those with ERD demonstrate a highly variable and unpredictable symptomatic response rate to antireflux treatment[1].

Cell replication of basal layers is hypothesized to be one of the causes implicated in the resistance of the mucosa and structural epithelial defense. In previous investigations, we have demonstrated that, in patients with GERD, the number of proliferating cells, evaluated by Ki-67 immunostaining, was reduced in esophageal mucosa exposed to chronic acid-peptic insult[2,3]. Two reasonable hypotheses can be suggested to explain the reduced epithelial proliferation activity observed in GERD: (1) chronic cell damage induced by GER determines a reduction in the proliferation rate of esophageal epithelium; or (2) a constitutive lower capacity for cell proliferation brings a major susceptibility to damage induced by GER.

Our findings are in contrast to the results of a recent study[4] on the cell proliferation of squamous epithelium in GERD. This study has shown a significantly higher number of proliferating cells in GERD patients compared with that in controls, as evaluated by Ki-67 immunostaining.

To elucidate the different proliferation in NERD and ERD patients, the present study evaluated squamous epithelial cell proliferation in patients with GERD, in comparison with NERD, by measuring the S-phase fraction using the bromodeoxyuridine labeling index (BrdU-LI), and by quantifying the expression of cyclins A and D, which are associated with cell cycle progression.

MATERIALS AND METHODS

Study design

Fifty consecutive patients with GERD-related symptoms were evaluated for enrolment into the study. Inclusion criteria were the presence of typical symptoms (heartburn and/or regurgitation) for at least 1 year (frequency was > 2 times/wk) and abnormal 24-h pH parameters and symptom-association probability (SAP). Exclusion criteria were patients with esophageal or gastric malignancy or histologically proven Barrett's esophagus, gastric or duodenal ulcer, previous esophageal or gastric surgery, extra-esophageal symptoms, patients taking antisecretory or prokinetic drugs at least 30 and 15 d before the procedure, respectively. Forty-six patients (mean age 45.2 ± 13.4 years, range 22-78 years; 20 men) fulfilled the inclusion/exclusion criteria and were evaluated. All these patients underwent standard endoscopy and biopsy for histological evaluation. Twenty-four had an apparently normal esophageal mucosa at endoscopy (NERD), whereas 22 had ERD. None of the patients had received cyclical therapy with proton pump inhibitors (PPIs) (not more than 8 wk in the past year). This study was single-blinded for the pH, histological, immunostaining and real-time reverse transcription polymerase chain reaction (RT-PCR) evaluations.

The frequency and intensity of symptoms and their impact on quality of life were registered using a structured and validated questionnaire for the diagnosis of GERD[5], and patients with a score > 3.1 were considered positive. For real-time RT-PCR, only 34 patients (mean age 47.08 ± 16.04 years, range 22-73 years; 16 men) were evaluable (Figure 1). Twenty-one had an apparently normal esophageal mucosa at endoscopy (NERD), whereas 13 had ERD (Table 1).

Patients gave written informed consent to participate in the study, which was approved by the local research ethical committee.

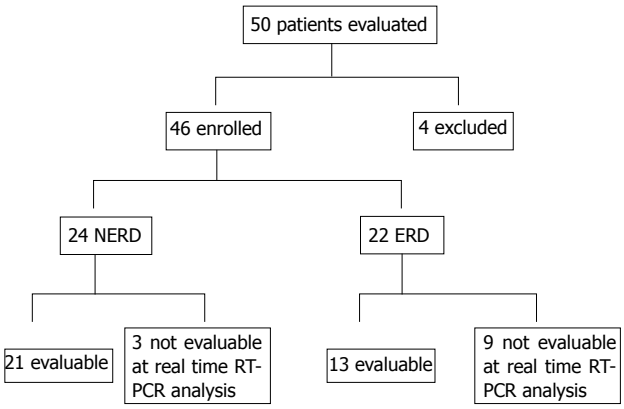

Figure 1 Study profile. RT-PCR: Reverse transcription polymerase chain reaction; NERD: Non-erosive reflux disease; ERD: Erosive reflux disease.

Table 1 Demographic, endoscopic, histological and 24-h esophageal pH monitoring data of the studied population

	NERD	ERD
No. of subjects	21	13
Sex (M/F)	9/12	7/6
Mean age ± SD (yr) (range)	44.2 ± 14.9 (22-73)	54.4 ± 15.7 (29-78)
Endoscopy		
Normal	21	0
A	0	0
B	0	7
C	0	6
D	0	0
Histology		
Normal	21	12
Mild	0	1
Moderate	0	0
Severe	0	0
24-h pH monitoring		
Mean % of acid exposure time ± SD	10.4 ± 1.3	10.7 ± 1.4
Mean number of acid reflux events ± SD	126 ± 20	128 ± 22

M: Male; F: Female; NERD: Non-erosive reflux disease; ERD: Erosive reflux disease.

Twenty-four-hour ambulatory pH monitoring

Every patient underwent 24-h esophageal pH monitoring according to standard methodology. To define better the localization of the lower esophageal sphincter (LES) and upper esophageal sphincter (UES), esophageal manometry was performed before pH monitoring, with a water-perfused catheter that incorporated three distal openings, radially oriented for LES pressure recording, and three side-hole recording sites at 5, 10 and 15 cm above the distal openings. Multichannel 24-h pH monitoring was performed using two probes, with one and two antimony sensors, respectively, with a separate skin reference (Zinetics Medical Inc., Salt Lake City, UT, United States). In accordance with manometric findings, the three pH sensors were placed at the gastric level, at 5 cm above the LES and 10 cm below the UES, respectively. Data were stored on a single portable digital recorder (Digitrapper pH 200; Medtronic, Minneapolis, MN, United States). Before each study, the pH probe was calibrated in buffer solutions of pH 7 and pH 1.

During the test day, meal time and composition were standardized. The reflux parameters were assessed according to Johnson and DeMeester[6]. Of these, only the percentage of time spent at pH < 4.0 over 24 h was evaluated. The pH testing was considered abnormal if pH < 4.0 was present for > 5% of the total 24 h. The SAP was calculated according to Weusten *et al*[7] and was considered positive if it exceeded 95%.

Endoscopic evaluation

Patients underwent upper gastrointestinal (GI) endoscopy (videogastroscope Olympus GIF 160) after sedation by intravenous midazolam (2.5 mg), to assess the presence or absence of erosions.

The Los Angeles classification was used to grade esophagitis[8]. In each subject, eight specimens were taken with standardized biopsy forceps (Olympus FB 24K), from each of the four quadrants, two bites from each quadrant, 5 cm above the squamous-columnar junction (SCJ), from macroscopically intact (non-eroded) esophageal mucosa. The SCJ (or Z-line) was defined as the border between gastric glandular and esophageal squamous epithelium, and it roughly corresponded to the proximal edge of the gastric folds.

Of the eight biopsies taken during endoscopy from each patient, two were used for total RNA extraction, and two for BrdU labeling. Four were oriented to appropriate cellulose acetate supports (Endofilters Bioptica, Milan, Italy), fixed in 4% buffered formalin, and embedded in paraffin, for processing by hematoxylin-eosin for histological and immunohistochemical analysis.

Histological evaluation

Four-micrometer-thick serial sections were cut from each paraffin block and stained with hematoxylin-eosin. For each case, whole longitudinally sectioned samples were examined. Esophagitis was identified and graded according to the classification of Ismail-Beigi *et al*[9]: (1) the degree of basal cell hyperplasia, expressed as a percentage of epithelial thickness: none (0%-15%), mild (16%-33%), moderate (34%-67%), severe (> 67%); (2) presence or absence of papillary zone elongation, determined by calculating papillary length as a percentage of epithelial thickness: absent (0%-67%) and present (> 67%); and (3) density of neutrophil and eosinophil infiltration: none (0/high power field), mild (1-2/high power field), moderate 3-10/high power field) and severe (> 10/high power field). The area of one high power field was 0.229 mm.

In vitro BrdU incorporation and immunohistochemical evaluation of S-phase cells

Each biopsy was immersed in 5 mL RPMI 1640 containing non-essential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (FBS) supplemented with 160 μmol/L BrdU (Sigma-Aldrich, St Louis, MO, United States) and incubated for 4 h at 37 ℃ in a 5% CO_2/air incubator. Tissues were rapidly rinsed with three washes of cold phosphate buffered saline (PBS), fixed in 10% buffered formalin, and embedded in paraffin. Sections were cut from each paraffin block and picked up on poly-L-lysine-coated slides. Sections were dewaxed, hydrated through decreasing concentrations of ethanol, rinsed in distilled water, and autoclaved in 10 mmol/L sodium citrate buffer (pH 6.0) at 120 ℃ for 21 min for antigen retrieval. After cooling and washing, the endogenous peroxidase activity was quenched using 3% hydrogen peroxide in absolute methanol for 10 min at room temperature. Sections were incubated with primary mouse anti-BrdU antibody (Bu20a; Abcam, Cambridge, United Kingdom) diluted in 1% bovine serum albumin in PBS overnight at 4 ℃, using appropriate dilutions.

Sections were processed according to a non-biotin amplified method (NovoLink™ Polymer Detection System; Novocastra Laboratories, Newcastle Upon Tyne, United Kingdom) and counterstained with hematoxylin.

Quantitative analysis of BrdU immunostaining was performed on contiguous field visualized on the color monitor of a personal computer equipped with a 3 CCD (charge-couple device) color video camera (KY F55B; JVC, Pinebrook, NJ, United States) connected to a light microscope (Leitz DIAPLAN, Wetzlar, Germany). For each case, whole longitudinally sectioned samples were examined. Samples that did not contain at least 1000 cells were excluded. Quantitative evaluation was only carried out on portions of epithelium in between vertically sectioned stromal papillae, and corresponding to 100 μm from the basal layer. BrdU-LI was defined as the ratio of BrdU-positive nuclei to the total number of epithelial cells, and was expressed as a percentage.

Esophageal epithelial cell isolation, RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from esophageal epithelial cells that were isolated as follows. Esophageal biopsy samples were washed in PBS and incubated in 0.5% collagenase type Ⅱ (C6885; Sigma-Aldrich) in 4-(2-hydroxyethyl)-1-

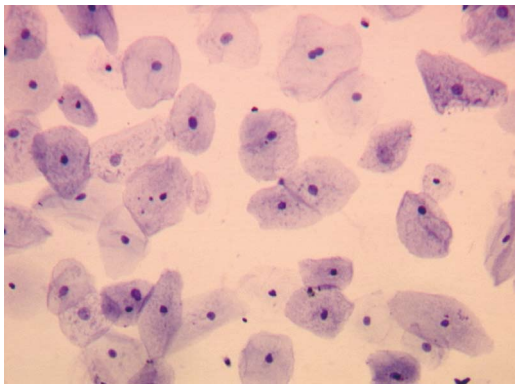

Figure 2 Cytological preparation from esophageal biopsy after epithelial cell isolation. Only epithelial cells were present. Toluidine blue staining (300 ×).

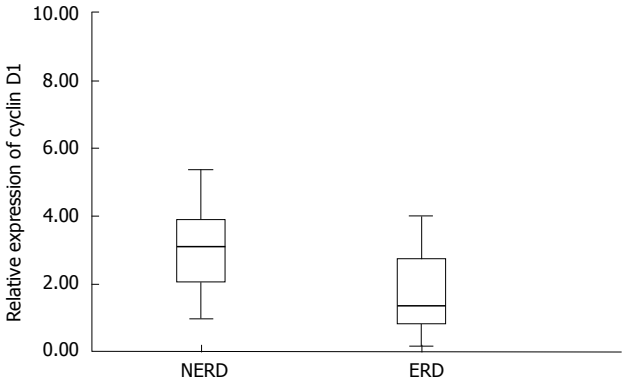

Figure 3 Box plots of relative expression of cyclin D1 mRNA by real-time RT-PCR analysis; median (bold line in box), and interquartile range (upper and lower lines of the box) in human esophageal mucosa of NERD and ERD patients ($P < 0.01$). RT-PCR: Reverse transcription polymerase chain reaction; NERD: Non-erosive reflux disease; ERD: Erosive reflux disease.

piperazineethanesulfonic acid (HEPES) buffer for 30 min at 37 ℃ in a shaking bath.

Collagenase activity was blocked by adding the same volume of 20% FBS in HEPES buffer. The digested material was re-suspended and passed through a 40-μm pore size cell strainer (BD Falcon™, Franklin Lakes, NJ United States) and centrifuged for 5 min at 800 *g*. Cells were washed in PBS and counted in a hemocytometer. From each biopsy sample, an average of 50 000 cells were recovered. Cell morphology was evaluated by seeding cells on poly-L-lysine-coated slides for 2 h at 37 ℃. Cells were fixed with 2% paraformaldehyde in PBS for 5 min and stained with 1% toluidine blue in distilled water for 1 min (Figure 2).

Total RNA was extracted from isolated esophageal epithelial cells using TRI Reagent (Ambion, Austin, TX, United States) according to the manufacturer's instructions. Whole cell RNA was quantified spectrophotometrically and 2 μg RNA for each sample was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States), following the manufacturer's protocol.

The relative expression of cyclin A (CCNA1), cyclin D1 (CCND1) and the housekeeping gene β-glucuronidase were evaluated by real-time polymerase chain reaction (PCR) performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using TaqMan Gene Expression Assay primers and probe kits (Assays catalog number Hs00927505 for CCNA1 and Hs00277039 for CCND1; Applied Biosystems). Cycling conditions were as follows: 50 ℃ for 2 min, 95 ℃ for 10 min, 40 cycles at 95 ℃ for 15 s, and 60 ℃ for 1 min. For each sample, three replicates were analyzed. The relative amounts of the transcripts were calculated with the $2^{-\Delta\Delta CT}$ method against aliquots from a single preparation of calibrator cDNA from the U2OS cell line.

Statistical analysis

Differences between groups were assessed by Student's *t* test. $P < 0.05$ was considered statistically significant. Data were analyzed with SPSS software (SPSS, Chicago, IL, United States).

RESULTS

At pH monitoring, the percentage time with esophageal pH < 4 in the two groups of patients (NERD and ERD) was 10.4% ± 1.3% and 10.7% ± 1.4%, respectively. No significant differences were found in the mean percentage time between the two groups.

Histological analysis showed that, among 13 patients affected by erosive esophagitis in endoscopic normal mucosa, 12 had a normal pattern, and 1 had mild esophagitis. None of the patients with NERD showed signs of esophagitis (Table 1).

Expression of cyclins A and D was evaluated by real-time RT-PCR from isolated epithelial cells. To check for purity of isolated cells, morphology was assessed after toluidine blue staining (Figure 2). In all samples evaluated, analysis of the isolated cell population revealed the presence of epithelial cells only.

Real-time RT-PCR analysis shows that, in patients with ERD, the relative expression of cyclin D1 mRNA, in esophageal epithelium, was strongly decreased in comparison with that of NERD patients (Figure 3). In particular, the relative expression of cyclin D1 mRNA in NERD epithelium was twofold higher, and showed elevated variability between patients, with respect to ERD epithelium. The relative expression of cyclin D1 mRNA ranged from 0.17 to 8.36 among all patients, with a mean (± SD) value of 2.41 ± 1.8. The mean (± SD) cyclin D1 value in 21 NERD patients was 3.44 ± 1.9, whereas in 13 ERD patients, it was 1.32 ± 0.87 ($P = 0.011$).

Only 25 of the 34 patients enrolled were evaluable for real-time RT-PCR analysis of cyclin A mRNA (18 NERD and 7 ERD). The relative expression of cyclin A mRNA ranged from 0 to 8.13 among all patients with a mean (± SD) value of 1.84 ± 2.59. Real-time RT-PCR analysis showed that, in patients with ERD, the relative expression of cyclin A mRNA in esophageal epithelium was decreased in comparison with that in NERD patients (Figure 4). In particular, the mean (± SD) cyclin A value of NERD patients was 2.31 ± 2.87, whereas in ERD patients, it was 0.66 ± 1.11. Despite the fact that

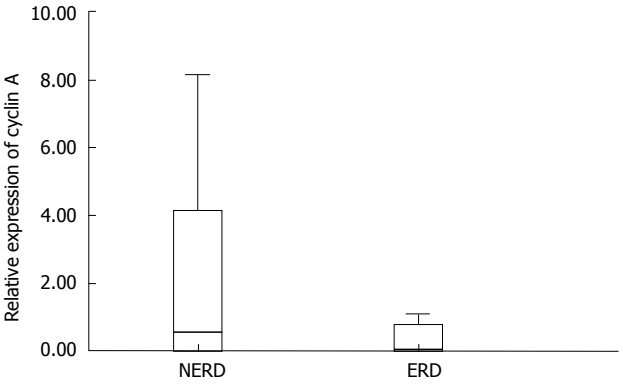

Figure 4 Box plots of relative expression of cyclin A mRNA by real-time RT-PCR analysis values; median (bold line in box), and interquartile range (upper and lower lines of the box) in human esophageal mucosa of NERD and ERD patients. RT-PCR: Reverse transcription polymerase chain reaction; NERD: Non-erosive reflux disease; ERD: Erosive reflux disease.

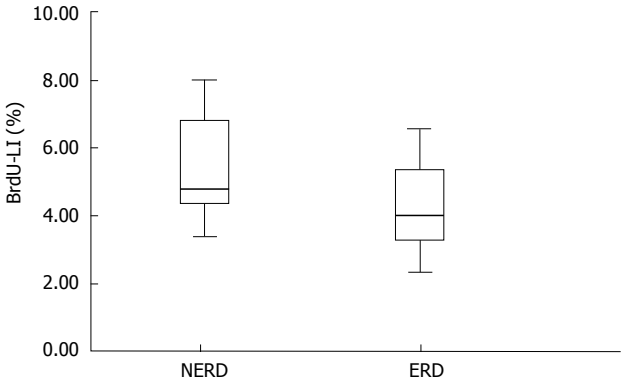

Figure 5 Box plots of BrdU-LI analysis values, median (bold line in the box), and interquartile range (upper and lower lines of the box) in human esophageal mucosa of NERD and ERD patients. NERD: Non-erosive reflux disease; ERD: Erosive reflux disease.

the relative expression of cyclin A mRNA in NERD epithelium was fourfold higher than in ERD epithelium, the difference between the two groups was not statistically significant ($P = 0.158$); both for the low number of cases evaluated, in particular in the ERD group, and for the high variability of the values relative to NERD patients.

Twelve patients were evaluable for BrdU analysis. BrdU-LI ranged from 2.33% to 8%, with a mean (± SD) value of 4.95% ± 1.67%. The mean BrdU-LI of the NERD patients ($n = 7$) was 5.42 % ± 1.68%, whereas in ERD patients ($n = 5$), it was 4.3% ± 1.59% (Figure 5). Once again, NERD epithelium showed a greater number of BrdU-positive cells than ERD epithelium did, but the difference between the two groups was not statistically significant ($P = 0.272$).

DISCUSSION

In the present study, we evaluated a series of esophageal biopsies to define the proliferation activity of the epithelium in patients with erosive or non-erosive GERD. In previous investigations, we have demonstrated that, in patients with GERD, cell proliferation evaluated by MIB1 immunostaining was reduced in esophageal mucosa exposed to chronic acid-peptic insult[2,3]. In particular, patients with NERD and ERD showed a decrease in cell proliferation to 50% and 75%, respectively, compared to normal subjects[2].

In contrast to our results, Mastracci *et al*[4] have found that MIB1 immunostaining of GERD patients is significantly greater than in controls. These different data might reflect different sampling conditions that could influence the proliferating activity of the epithelial cells. In particular, Mastracci and co-workers have evaluated specimens that were taken from 2-4 cm to the Z line, and observed a progressive decrease in the Ki-67 LI by increasing the distance from the Z line.

Feagins *et al*[10] have shown that multiple acid exposures decrease cell proliferation in non-neoplastic, telomerase-immortalized Barrett's cell lines. This decrease in cell proliferation is the result of a delay in cell cycle progression that is mediated by p53. In agreement with these results, we have recently demonstrated that, in patients with ERD and NERD, long-term PPI therapy increases esophageal cell proliferation[3]. These data confirm that acid-peptic insults have an antiproliferative effect on esophageal epithelial cells.

In the present study, only patients with at least a 1-year history of GERD were included. Upper endoscopy was performed and biopsies were taken only in apparently normal mucosa at 5 cm above the Z-line. In this way, we studied the behavior of the mucosa exposed to chronic acid insult, but far from erosions, and especially, from reparative changes secondary to the lack of the superficial mucosa, where basal cell hyperplasia has been reported[11], which can be characterized by increased proliferative activity.

Regardless of these considerations, in the present study, we evaluated proliferative activity of the epithelium in patients with erosive and non-erosive GERD. For this purpose, three proliferation markers were assessed: cyclins A and D relative expression, evaluated by real-time RT-PCR, and *in vitro* BrdU incorporation for immunohistochemical detection of S-phase cells in histological samples.

Cyclins are a family of proteins involved in cell cycle regulation. Cyclin expression rises and falls at various stages of the cell cycle, thus activating specific cyclin dependent kinases (CDKs), which, by phosphorylation of multiple substrates, control a number of critical steps in cell cycle progression[12]. Cyclin D1 is encoded by the *CCND1* gene located on chromosome 11q13, and in association with CDK4 or CDK6, regulates the transition from G1 to S phase[13,14]. It is synthesized in response to extracellular mitogenic signals and is maximally expressed in mid-to-late G1-phase[15]. Cyclin A, in association with CDK1 or CDK2, promotes the transition from G2 to M phase[16], and is expressed later in the cell cycle, during DNA replication, achieving its maximal levels during late S-phase[17,18]. Cyclins D1 and A are regarded

as specific markers of the G1 and S phases of the cell cycle, respectively. Therefore, in the present study, we evaluated their mRNA expression to assess the proliferative activity of esophageal epithelial cells. In previous investigations, we have in fact demonstrated that the relative expression of cyclin mRNA is directly related to the cell proliferation rate in breast cancer specimens[19]. Moreover, to identify S-phase cells specifically, in the present study, DNA-synthesizing cells were detected *in situ* after BrdU incorporation by immunohistochemical analysis with anti-BrdU antibodies.

Our results demonstrated that cyclin D, as a marker of G1 phase, was significantly higher in NERD compared to ERD. Also the S-phase markers evaluated in our study (cyclin A and BrdU) were higher in NERD compared to ERD, although in this case, because of the small number of evaluable samples, the difference was not statistically significant. The reduction in the number of samples analyzed for cyclin A compared to those analyzed for cyclin D was due to the fact that the thickness of the epithelium in ERD was significantly reduced, and therefore, in these samples, it was not always possible to isolate a sufficient number of epithelial cells for molecular analysis.

The limitation of this study was the small number of patients, but this is believed to be the first study to evaluate, at the molecular level, esophageal epithelial cells. This method is clean but it creates considerable tissue loss.

In conclusion, the present study confirmed our previous results regarding the reduction of epithelial proliferative activity in ERD compared with NERD patients. Besides, this study supports the previous data on an antiproliferative effect of acid-peptic injury in esophageal cell epithelium, and reinforces the idea that individuals who develop ERD might be genetically characterized by weaker epithelial cell proliferation. On the other hand, patients with more efficient epithelial proliferation capability could have a lower probability of developing macroscopic mucosal lesions when stressed by acid and pepsin. Further studies are required to understand better the mucosal defense mechanisms, and in particular, those controlling the cellular proliferative activity of esophageal mucosa.

COMMENTS

Background

Cell replication in basal layers has been suggested as one of the causes of mucosal resistance and structural epithelial defense. To elucidate better the different proliferative activity between non-erosive reflux disease (NERD) and erosive reflux disease (ERD), the authors evaluated a series of molecular and immunohistochemical markers of cell proliferation in squamous epithelial cells of patients with gastroesophageal reflux disease (GERD).

Research frontiers

In previous investigations, the authors have demonstrated that, in patients with GERD, cell proliferation evaluated by MIB1 immunostaining is reduced in esophageal mucosa exposed to chronic acid-peptic insult, in contrast with other studies. These different data might reflect different sampling conditions that could influence the proliferating activity of the epithelial cells.

Innovations and breakthroughs

The present study confirmed the previous results with regard to reduction of epithelial cell proliferative activity in ERD compared with NERD patients. It supports previous data on an antiproliferative effect of acid-peptic injury in esophageal cell epithelium, and reinforces the idea that individuals who develop ERD might be genetically characterized by a weaker proliferating epithelial cell capability.

Applications

This paper shows that patients with more efficient epithelial proliferative capability could have a lower probability of developing macroscopic mucosal lesions when stressed by acid and pepsin.

Peer review

The paper by Dr. Calabrese and colleagues discusses activity of cells in esophageal squamous epithelium in patients with NERD and ERD. This is a very well written manuscript that contributes to the knowledge of the molecular mechanisms in patients with GERD.

REFERENCES

1 **Hershcovici T**, Fass R. Nonerosive Reflux Disease (NERD) - An Update. *J Neurogastroenterol Motil* 2010; **16**: 8-21

2 **Calabrese C**, Trerè D, Fabbri A, Cenacchi G, Vici M, Derenzini M, Di Febo G. Endoscopic appearance of GERD: putative role of cell proliferation. *Dig Liver Dis* 2007; **39**: 713-719

3 **Calabrese C**, Treré D, Liguori G, Gabusi V, Vici M, Cenacchi G, Derenzini M, Di Febo G. Esophageal cell proliferation in gastroesophageal reflux disease: clinical-morphological data before and after pantoprazole. *World J Gastroenterol* 2009; **15**: 936-941

4 **Mastracci L**, Grillo F, Zentilin P, Spaggiari P, Dulbecco P, Pigozzi S, Savarino V, Fiocca R. Cell proliferation of squamous epithelium in gastro-oesophageal reflux disease: correlations with clinical, endoscopic and morphological data. *Aliment Pharmacol Ther* 2007; **25**: 637-645

5 **Pacini F**, Calabrese C, Cipolletta L, Valva MD, Russo A, Savarino V, Vigneri S. Burden of illness in Italian patients with gastro-oesophageal reflux disease. *Curr Med Res Opin* 2005; **21**: 495-502

6 **Johnson LF**, Demeester TR. Twenty-four-hour pH monitoring of the distal esophagus. A quantitative measure of gastroesophageal reflux. *Am J Gastroenterol* 1974; **62**: 325-332

7 **Weusten BL**, Roelofs JM, Akkermans LM, Van Berge-Henegouwen GP, Smout AJ. The symptom-association probability: an improved method for symptom analysis of 24-hour esophageal pH data. *Gastroenterology* 1994; **107**: 1741-1745

8 **Armstrong D**, Bennett JR, Blum AL, Dent J, De Dombal FT, Galmiche JP, Lundell L, Margulies M, Richter JE, Spechler SJ, Tytgat GN, Wallin L. The endoscopic assessment of esophagitis: a progress report on observer agreement. *Gastroenterology* 1996; **111**: 85-92

9 **Ismail-Beigi F**, Horton PF, Pope CE. Histological consequences of gastroesophageal reflux in man. *Gastroenterology* 1970; **58**: 163-174

10 **Feagins LA**, Zhang HY, Hormi-Carver K, Quinones MH, Thomas D, Zhang X, Terada LS, Spechler SJ, Ramirez RD, Souza RF. Acid has antiproliferative effects in nonneoplastic Barrett's epithelial cells. *Am J Gastroenterol* 2007; **102**: 10-20

11 **Funch-Jensen P**, Kock K, Christensen LA, Fallingborg J, Kjaergaard JJ, Andersen SP, Teglbjaerg PS. Microscopic appearance of the esophageal mucosa in a consecutive series of patients submitted to upper endoscopy. Correlation with gastroesophageal reflux symptoms and macroscopic findings. *Scand J Gastroenterol* 1986; **21**: 65-69

12 **Cordon-Cardo C**. Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia. *Am J Pathol* 1995; **147**: 545-560

13 **Baldin V**, Lukas J, Marcote MJ, Pagano M, Draetta G. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev* 1993; **7**: 812-821

14 **Sherr CJ**. The Pezcoller lecture: cancer cell cycles revisited. *Cancer Res* 2000; **60**: 3689-3695
15 **Sherr CJ**. Cancer cell cycles. *Science* 1996; **274**: 1672-1677
16 **Fotedar R**, Fotedar A. Cell cycle control of DNA replication. *Prog Cell Cycle Res* 1995; **1**: 73-89
17 **Pines J**, Hunter T. Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. *Cell* 1989; **58**: 833-846
18 **Pines J**, Hunter T. Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. *Nature* 1990; **346**: 760-763
19 **Montanaro L**, Vici M, Donati G, Ceccarelli C, Santini D, Treré D, Derenzini M. Controversial relationship between the expression of the RB pathway components and RB protein phosphorylation in human breast cancer. *Histol Histopathol* 2007; **22**: 769-775

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BRIEF ARTICLE

Liver hemangioma and vascular liver diseases in patients with systemic lupus erythematosus

Annalisa Berzigotti, Marilena Frigato, Elena Manfredini, Lucia Pierpaoli, Rita Mulè, Carolina Tiani, Paola Zappoli, Donatella Magalotti, Nazzarena Malavolta, Marco Zoli

Annalisa Berzigotti, Lucia Pierpaoli, Carolina Tiani, Paola Zappoli, Donatella Magalotti, Marco Zoli, Department of Internal Medicine, Nephrology and Ageing, University of Bologna, 40138 Bologna, Italy
Marilena Frigato, Elena Manfredini, Rita Mulè, Nazzarena Malavolta, UOS Reumatologia, Policlinico S, Orsola-Malpighi, 40138 Bologna, Italy
Author contributions: Berzigotti A and Frigato M designed the research; Berzigotti A, Manfredini E, Pierpaoli L, Mulè R, and Magalotti D performed the research; Berzigotti A and Tiani C analyzed the data; Berzigotti A and Zappoli P wrote the paper; Malavolta N and Zoli M supervised the entire work.
Supported by Department of Internal Medicine, Nephrology and Ageing of University of Bologna, Italy
Correspondence to: Marco Zoli, MD, Department of Internal Medicine, Nephrology and Ageing, University of Bologna, Policlinico S Orsola-Malpighi, Via Albertoni 15, 40138 Bologna, Italy. marco.zoli@unibo.it
Telephone: +39-051-6362211 Fax: +39-051-6362210
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Abstract

AIM: To investigate whether systemic lupus erythematosus (SLE) is associated with benign focal liver lesions and vascular liver diseases, since these have been occasionally reported in SLE patients.

METHODS: Thirty-five consecutive adult patients with SLE and 35 age- and sex-matched healthy controls were evaluated. Hepatic and portal vein patency and presence of focal liver lesions were studied by colour-Doppler ultrasound, computerized tomography and magnetic resonance were used to refine the diagnosis, clinical data of SLE patients were reviewed.

RESULTS: Benign hepatic lesions were common in SLE patients (54% *vs* 14% controls, $P < 0.0001$), with hemangioma being the most commonly observed lesion in the two groups. SLE was associated with the presence of single hemangioma [odds ratios (OR) 5.05; 95% confidence interval (CI) 1.91-13.38] and multiple hemangiomas (OR 4.13; 95% CI 1.03-16.55). Multiple hemangiomas were associated with a longer duration of SLE (9.9 ± 6.5 *vs* 5.5 ± 6.4 years; $P = 0.04$). Imaging prior to SLE onset was available in 9 patients with SLE and hemangioma, showing absence of lesions in 7/9. The clinical data of our patients suggest that SLE possibly plays a role in the development of hemangioma. In addition, a Budd-Chiari syndrome associated with nodular regenerative hyperplasia (NRH), and a NRH associated with hepatic hemangioma were observed, both in patients hospitalized for abdominal symptoms, suggesting that vascular liver diseases should be specifically investigated in this population.

CONCLUSION: SLE is associated with 5-fold increased odds of liver hemangiomas, suggesting that these might be considered among the hepatic manifestations of SLE.

Key words: Colour-Doppler ultrasound; Portal hypertension; Rheumatic diseases; Portal vein; Hepatic vein thrombosis

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INTRODUCTION

The spectrum of liver disease in systemic lupus erythematosus (SLE) patients is wide, ranging from benign conditions (such as benign liver lesions and mild chronic hepatitis), to aggressive and potentially lethal disorders (such as vascular liver diseases, including portal and hepatic veins thrombosis, nodular regenerative hyperplasia and idiopathic portal hypertension)[1,2]. Benign focal liver lesions, such as cavernous hemangioma, have been reported in SLE[3,4], and vascular liver diseases in SLE have been frequently observed in autoptic series[5], suggesting an association between these disorders, but data in living SLE patients are scarce and rely on isolated case reports.

Color-Doppler ultrasonography (CDUS) is the initial imaging technique used to screen patients with suspected liver disease, since it allows a non-invasive, real-time evaluation of abdominal organs and vessels, which is repeatable, easy to perform and inexpensive compared with other techniques. Ultrasound allows an accurate identification and characterization of focal hepatic lesions[6], and of portal vein and hepatic veins patency; in cases of portal or hepatic vein thrombosis, CDUS accuracy is similar to that of computerized tomography (CT)[7-13].

This case-control study was aimed at investigating, *via* CDUS, whether SLE is associated with focal lesions and vascular liver diseases.

MATERIALS AND METHODS

Patients and controls

We prospectively included in the present study all consecutive patients with an established diagnosis of SLE, namely at least 4 criteria among those published in the guidelines by the American College of Rheumatology[14]. Exclusion criteria were the presence of previously recognized liver disease from alcohol, hepatitis B virus (HBV) or hepatitis C virus (HCV) virus or autoimmune causes, and personal history of malignancy.

Thirty subjects with SLE, normal aspartate transaminase (AST)/alanine transaminase (ALT) and no recognized liver disease observed at our outpatient Unit, and 5 patients with SLE requiring hospitalization for any cause at our Unit over 24 mo were consecutively enrolled.

Thirty-five age and sex-matched healthy controls without SLE were included. Controls were consecutively recruited among subjects referred for an abdominal ultrasound examination to the Ultrasound Laboratory of our Unit for the following reasons: routine screening (n = 12), follow-up of renal or hepatic cyst (n = 11), evaluation of gallbladder lithiasis (n = 6), and abdominal discomfort (n = 6). Healthy state was ensured by specific questions on liver, heart, lung or renal diseases, history of malignancy, and chronic medication intake. Subjects with history of any of these conditions were excluded.

This study was approved by the Senior Staff Committee of University Hospital, a board which regulates non-interventional studies and is comparable to an Institutional Review Board. The nature of the study was explained to the patients and controls, and a written informed consent was obtained in each case, according to the principles of the Declaration of Helsinki (revision of Edinburgh 2000).

Ultrasound-Doppler examination

After an overnight fast, patients and controls were entered in the ultrasound examination room and invited to remain in the supine position for 10 min. Thereafter, an abdominal color-Doppler ultrasonography (CDUS) examination was performed by an experienced operator by using last-generation duplex equipment (Esaote Ansaldo AU Technos, Genoa, Italy) with a 4.5-7 MHz convex probe provided by a color, power and pulsed Doppler device.

Liver parenchyma and portal and hepatic veins patency were systematically evaluated. Location, number and size of the focal liver lesions were recorded. If present, they were diagnosed as[6]: (1) typical liver hemangioma: round-shaped, hyperechoic lesion with sharp margins, up to 4 cm in size. No Doppler signal inside the lesion; (2) atypical liver hemangioma: size > 4 cm; hypoechoic lesion or heterogeneous echopattern. No Doppler signal inside the lesion; and (3) focal nodular hyperplasia (FNH): hypo-iso- or slightly hyperechoic lesion < 3 cm with sharp margins and typical color-Doppler findings: central feeding artery and spoke wheel centrifugal vascular pattern.

When ultrasound (US) suggested atypical hemangioma, FNH or lesions of uncertain nature, a definite diagnosis was achieved by magnetic resonance imaging or by multislice CT scan.

Clinical and laboratory data of SLE patients

Routine clinical and laboratory data were collected, and data on the duration of the disease and its treatment were recorded. Systemic lupus erythematosus disease activity index (SLEDAI)[15] and Systemic lupus international collaborating clinics/American college of rheumatology damage index for systemic lupus erythematosus (SLICC/ACR)[16] were calculated.

Statistical analysis

Statistical analysis was performed by SPSS 12.0 statistical package (SPSS Inc., Chicago, IL, United States). All results are expressed as mean ± SD. Comparisons between cases and controls were done by Student's t test for unpaired data for continuous normally distributed variables, and by χ^2 test for frequencies; Mann-Whitney test was used for non-normally distributed continuous variables. The strength of the association between SLE and the conditions in study were estimated by odds ratios (OR) and their 95% confidence interval (CI). A P value of < 0.05 was considered statistically significant.

RESULTS

Focal hepatic lesions in SLE patients and controls

Table 1 summarizes the main characteristics of the stud-

Table 1 Main clinical, laboratory and ultrasound characteristics of the patients included in the study (*n* = 35)

	Overall (*n* = 35)	Outpatients (*n* = 30)	Hospitalized (*n* = 5)
Age (yr)	50 ± 20	50 ± 17	51 ± 29
Gender (M/F)	2/33	1/29	1/4
SLE duration (yr)	6.9 ± 7.0	6.5 ± 6.6	9.6 ± 9.8
LAC positivity	8/35	7/30	1/5
SLEDAI[1]	8.9 ± 4.2	9.1 ± 4.3	7.6 ± 2.8
ACR/SLICC[1]	2.1 ± 1.6	2.1 ± 1.7	2.2 ± 1.3
Acrocyanosis[1]	24/33	18/28	4/5
Steroid treatment[1]	26/33	22/28	4/5
Duration of steroid therapy (yr)[1]	6.4 ± 6.3	5.9 ± 5.7	9.5 ± 9.4
AST (U/L)	22 ± 9	20 ± 5	34 ± 18
ALT (U/L)	22 ± 9	18 ± 8	24 ± 13
Bilirubin (mg/dL)	0.6 ± 0.4	0.5 ± 0.2	1.1 ± 0.8
GGT (U/L)	32 ± 40	22 ± 20	67 ± 72
ALP (U/L)	190 ± 105	171 ± 78	247 ± 159
Thrombosis of hepatic veins	1	0	1
Normal liver echopattern	9	8	1
NRH	2	0	2
Single hepatic hemangioma	12	9	3
Multiple hepatic hemangioma	7	7	0
Atypical hemangioma	5	2	3
FNH	2	2	0

[1]Data available in 33 patients. M: Male; F: Female; SLE: Systemic lupus erythematosus; LAC: Lupus anticoagulant; SLEDAI: Systemic lupus erythematosus disease activity index; ACR: American college of rheumatology damage index for systemic lupus erythematosus; SLICC: Systemic lupus international collaborating clinics; AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase; NRH: Nodular regenerative hyperplasia; FNH: Focal nodular hyperplasia.

ied patients with SLE.

As shown, 19 patients (54.2%) showed one or more benign focal liver lesion. Hemangioma was the most frequent diagnosis, being observed in 19 cases (54.2%). FNH was observed in 2 cases (5.7%), and in both cases was associated with hemangioma.

Hemangiomas were observed both in outpatients and in hospitalized patients. Among hospitalized patients, a single hemangioma was seen in association with nodular regenerative hyperplasia (NRH); in two additional patients with no abdominal symptoms, admitted for fever in one case and for polyarthralgia in one case, a single hepatic hemangioma of large size in the right lobe (4.5 cm and 6 cm) was diagnosed.

Among outpatients, 16 (53.3%) had focal liver lesions; 9 had a single hepatic hemangioma (in two cases with atypical hypoechogenic US aspect requiring CT scan; size 10-22 mm), and 7 (20% of patients of the whole series and 23.3% of outpatients) had multiple typical hepatic hemangiomas (number 2-9; size 10-22 mm) (Figure 1).

Hemangiomas did not demonstrate a preferential location inside the liver, being equally distributed in all segments.

Two patients with multiple hepatic hemangiomas had a FNH associated (confirmed by magnatic resonance imaging). Furthermore, one patient showed previously undiagnosed Caroli disease of left liver lobe associated with a single atypical hemangioma of the right lobe. These findings were confirmed by abdominal MR.

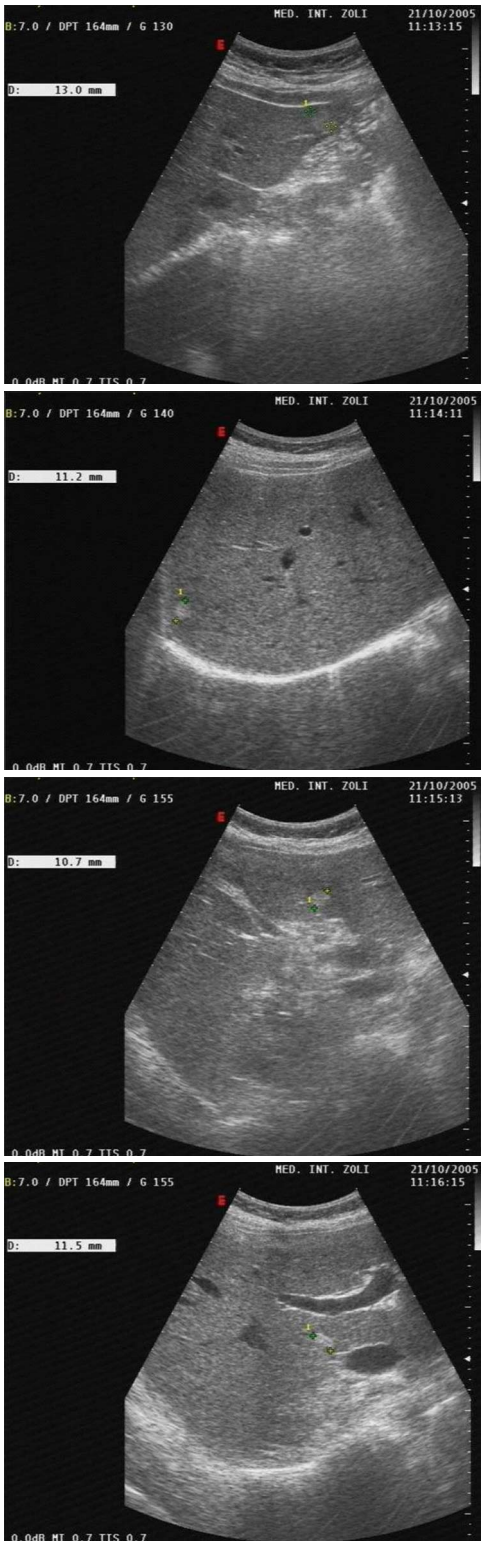

Figure 1 Ultrasonographic appearance of multiple hemangiomas in one of the studied patients. In this 47-year-old woman, 4 hepatic lesions were detected in 4 different segments of the liver.

Five control subjects (14%) were diagnosed of hemangioma. Two of them had multiple lesions (2 lesions in

Table 2 Clinical and laboratory characteristics of systemic lupus erythematosus patients according to the presence and number of hepatic hemangioma

	Patients with HH (*n* = 19)	Single HH (*n* = 12)	Multiple HH (*n* = 7)	Patients without HH (*n* = 16)
Age (yr)	51 ± 16	52 ± 17	52 ± 17	49 ± 18
Gender (M/F)	2/17	2/10	0/7	0/16
SLE duration (yr)	7.1 ± 6.4	5.5 ± 6.4	9.9 ± 6.5[a]	6.8 ± 6.1
Clinical flares (*n*)	3.5 ± 1.8	2.9 ± 1.4	4.2 ± 2.1	2.7 ± 1.4
SLEDAI[1]	8.3 ± 4.3	8.9 ± 5.1	7.4 ± 2.6	10.0 ± 4.3
ACR/SLICC[1]	2.3 ± 1.9	2.0 ± 1.7	3.0 ± 2.0	1.9 ± 1.4
Acrocyanosis[1] (%)	94.4	100.0	85.7	43.8[b]
Steroid treatment (%)[1]	75.0	60.0	100.0	87.5
Yr of steroid Rx (yr)[1]	6.8 ± 6.2	8.2 ± 7.1	5.5 ± 5.6	6.6 ± 6.1
LAC positivity (%)	22.2	18.2	28.6	25.0
AST (U/L)	23 ± 11	25 ± 14	21 ± 4	21 ± 7
ALT (U/L)	18 ± 6	17 ± 6	20 ± 8	19 ± 11
Bilirubin (mg/dL)	0.7 ± 0.5	0.7 ± 0.7	0.7 ± 0.3	0.5 ± 0.2
GGT (U/L)	36 ± 54	42 ± 65	23 ± 9	29 ± 28
ALP (U/L)	213 ± 133	243 ± 134	169 ± 138	168 ± 65

[1]Data available in $n = 33$. [a]$P < 0.05$ *vs* patients with single hepatic hemangioma and [b]$P = 0.02$ *vs* patients with hepatic hemangioma. M: Male; F: Female; SLE: Systemic lupus erythematosus; SLEDAI: Systemic lupus erythematosus disease activity index; SLICC: Systemic lupus international collaborating clinics; ACR: American college of rheumatology damage index for systemic lupus erythematosus; LAC: Lupus anticoagulant; AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase; HH: Hepatic hemangioma.

both cases).

SLE was associated with the presence of single hemangioma (OR 5.05; 95% CI 1.91-13.38) and multiple hemangiomas (OR 4.13; 95% CI 1.03-16.55).

Time-course of hemangioma in SLE

In order to ascertain whether hemangiomas formation was associated with SLE onset, we evaluated the clinical files of patients with liver hemangioma to identify imaging prior to SLE development. These data were found in 9/19 cases and consisted of US examination in 7 and CT scan in 2. In 7/9 cases imaging studies documented a normal liver, while in two cases a hemangioma was already present. In the two cases of pre-existent liver hemangioma, the lesion's size at the time of the present study was stable in one, and increased from 15 to 21 mm in one.

Association between clinical variables and liver hemangioma in SLE

We did not find any significant difference between patients with and without hemangiomas for laboratory parameters, age and prevalence of the main clinical manifestation of SLE (glomerulonephritis, neurological symptoms, pulmonary hypertension, and polyserositis) (Table 2).

We observed a significantly higher prevalence of acrocyanosis in patients with hemangioma compared with patients without focal liver lesions: 94% *vs* 47% ($P = 0.02$).

We evaluated whether the presence of liver hemangioma was associated with duration of SLE disease, activity of the disease at onset (SLEDAI), damage index at the time of US examination (SLICC) and number of clinical flares of SLE.

Multiple hepatic hemangiomas were associated with a longer duration of SLE disease (9.9 ± 6.5 *vs* 5.5 ± 6.4, $P = 0.049$). Patients with hepatic hemangiomas also showed a higher number of clinical flares, but the difference did not reach statistical significance.

As for treatment, the rate and duration of corticosteroid therapy for SLE were similar in patients with and without hemangioma.

Prevalence of vascular liver disease in SLE patients

We observed 2 cases of vascular liver diseases (5.7%). Both were identified in hospitalized patients with abdominal symptoms. The first patient (female, 39 year) was admitted for ascites and showed chronic Budd-Chiari syndrome with clinical and US signs of portal hypertension (namely small esophageal varices at endoscopy and enlargement of portal vein and spleen, and porto-collateral circulation at US), and NRH. A percutaneous biopsy was obtained and confirmed the radiological diagnosis. The second patient (female, 47 year) was admitted for a biliary colic, and CDUS identified a diffuse and severe alteration of liver echopattern associated with a 4 cm hyperechoic nodule. On CT scan she was diagnosed of probable NRH associated with a 4 cm hemangioma; portal and hepatic veins were patent. The patient refused liver biopsy.

DISCUSSION

The main result of the present study is the demonstration of an association between SLE and liver hemangioma. The prevalence of liver hemangioma was 54.2% in our SLE patients; this figure is more than twice the maximum expected in the general adult population (0.4%-20%)[17], and was significantly higher than the 14% observed in healthy control subjects. Accordingly, in this study SLE was associated with increased odds (5 fold increase) of hemangioma occurrence.

This data suggests that SLE may be directly implicated in the formation of benign vascular neoplasia. Some additional findings from our study support this hypothesis: hemangioma seemed to appear after SLE onset in 7 patients; it increased in size in one; hemangiomas were multiple in 40% of patients, and patients with multiple hemangiomas had a long-lasting disease.

Hepatic hemangioma is a benign vascular neoplasia of endothelial origin. Even if the pathogenetic mechanisms leading to its formation have not been fully elucidated yet, it has been proposed that hemangiomas are caused by unregulated angiogenesis due to an imbalance between angiogenic and angiostatic factors[18]. This hypothesis is supported by the observation that hemangiomas may increase in dimension over time during pregnancy and estrogen therapy[19], as a consequence of estrogens-enhanced neo-

angiogenesis[20].

It has been shown that SLE patients have increased circulating levels of estrogens[21] and other angiogenic factors such as vascular endothelial growth factor (VEGF) and IL-18, which are associated with activity of disease[22,23]. It could be speculated that an activation of angiogenesis might lead to liver hemangiomas formation in the course of the disease. Regrettably we lack evidence to support this hypothesis, since VEGF and other pro-angiogenic cytokines were not dosed in our patients.

In two patients of our series, hemangiomas were associated with FNH. FNH is a benign hepatocellular lesion which has been reported in 0.6%-3.0% of the general population[17]. It has been showed that FNH represents an abnormal adaptive responsive of liver parenchyma to local hemodynamic disturbances[24]. The association of hemangioma and FNH is frequent[25,26], and several authors have speculated that both lesions may have causative factors in common, including focal disturbance of the hepatic blood supply that somehow facilitates the hyperplastic development of these benign lesions[27].

In the present series of SLE patients, we found 2 cases of vascular diseases of the liver, which were diagnosed in patients hospitalized for abdominal symptoms (ascites and increase of hepatic enzymes, and suspect of biliary colic). Vascular diseases of the liver, such as hepatic and portal vein thrombosis, are life-threatening events occurring more often in patients with congenital or acquired prothrombotic condition[28]. SLE is a well recognized prothrombotic condition and vascular liver diseases have been reported in this setting[1,2]; our experience is in line with these previous reports, and suggests that US examination should be performed in cases of abdominal symptoms in SLE patients to specifically rule out vascular liver diseases.

The present study suffers from some limitations. We lack histological confirmation for the imaging findings since most lesions were found in asymptomatic patients, in whom biopsy was not performed. Still, in patients without chronic hepatic diseases, imaging techniques are considered sufficient to diagnose benign liver lesions[29].

The clinical observations from our series suggest an association between SLE and liver hemangioma formation. Future studies are needed to assess the mechanisms leading to this association.

In conclusion, this study shows that the liver is a frequent site of abnormal findings in SLE patients. SLE is associated with an increased likelihood of liver hemangiomas and multiple hepatic hemangiomas, which can be associated with FNH and vascular liver diseases. Vascular disorders can be found in patients with SLE, and should be actively looked for in SLE patients with abdominal symptoms.

COMMENTS

Background

Benign focal liver lesions, such as cavernous hemangioma, and vascular liver diseases have been reported in systemic lupus erythematosus (SLE), suggesting an association between these disorders.

Research frontiers

Only a few case reports and autoptic series have been published hitherto regarding the prevalence of benign liver lesions and vascular liver diseases in patients with SLE.

Innovations and breakthroughs

In this study, the authors found that the prevalence of hepatic hemangioma is very high in patients with SLE. Moreover, liver hemangiomas in this population were often multiple, and associated in some instance with focal nodular hyperplasia and vascular liver diseases.

Applications

The clinical observations from this study suggest an association between SLE and liver hemangioma formation. Vascular hepatic disorders can be found in patients with SLE, and should be actively looked for in SLE patients with abdominal symptoms.

Peer review

Although this study on liver findings in SLE patients does not lead to direct therapeutic consequences, it evaluates an interesting aspect of this disease Histological proof would of course be desirable, but in general, biopsy cannot be justified in this context.

REFERENCES

1 **van Hoek B**. The spectrum of liver disease in systemic lupus erythematosus. *Neth J Med* 1996; **48**: 244-253
2 **Youssef WI**, Tavill AS. Connective tissue diseases and the liver. *J Clin Gastroenterol* 2002; **35**: 345-349
3 **Maeshima E**, Minami Y, Sato M, Matsuda K, Uchiyama K, Goda M, Ueda H, Kida Y, Mune M. A case of systemic lupus erythematosus with giant hepatic cavernous hemangioma. *Lupus* 2004; **13**: 546-548
4 **Suzuki T**, Tsuchiya N, Ito K. Multiple cavernous hemangiomas of the liver in patients with systemic lupus erythematosus. *J Rheumatol* 1997; **24**: 810-811
5 **Matsumoto T**, Yoshimine T, Shimouchi K, Shiotu H, Kuwabara N, Fukuda Y, Hoshi T. The liver in systemic lupus erythematosus: pathologic analysis of 52 cases and review of Japanese Autopsy Registry Data. *Hum Pathol* 1992; **23**: 1151-1158
6 **Harvey CJ**, Albrecht T. Ultrasound of focal liver lesions. *Eur Radiol* 2001; **11**: 1578-1593
7 **Bargalló X**, Gilabert R, Nicolau C, García-Pagán JC, Ayuso JR, Brú C. Sonography of Budd-Chiari syndrome. *AJR Am J Roentgenol* 2006; **187**: W33-W41
8 **Finn JP**, Kane RA, Edelman RR, Jenkins RL, Lewis WD, Muller M, Longmaid HE. Imaging of the portal venous system in patients with cirrhosis: MR angiography vs duplex Doppler sonography. *AJR Am J Roentgenol* 1993; **161**: 989-994
9 **Janssen HL**, Garcia-Pagan JC, Elias E, Mentha G, Hadengue A, Valla DC. Budd-Chiari syndrome: a review by an expert panel. *J Hepatol* 2003; **38**: 364-371
10 **Ralls PW**, Johnson MB, Radin DR, Boswell WD, Lee KP, Halls JM. Budd-Chiari syndrome: detection with color Doppler sonography. *AJR Am J Roentgenol* 1992; **159**: 113-116
11 **Tanaka K**, Numata K, Okazaki H, Nakamura S, Inoue S, Takamura Y. Diagnosis of portal vein thrombosis in patients with hepatocellular carcinoma: efficacy of color Doppler sonography compared with angiography. *AJR Am J Roentgenol* 1993; **160**: 1279-1283
12 **Valla DC**. The diagnosis and management of the Budd-Chiari syndrome: consensus and controversies. *Hepatology* 2003; **38**: 793-803
13 **Van Gansbeke D**, Avni EF, Delcour C, Engelholm L, Struyven J. Sonographic features of portal vein thrombosis. *AJR Am J Roentgenol* 1985; **144**: 749-752
14 **Hochberg MC**. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; **40**: 1725
15 **Gladman DD**, Ibañez D, Urowitz MB. Systemic lupus ery-

thematosus disease activity index 2000. *J Rheumatol* 2002; **29**: 288-291
16 **Gladman D**, Ginzler E, Goldsmith C, Fortin P, Liang M, Urowitz M, Bacon P, Bombardieri S, Hanly J, Hay E, Isenberg D, Jones J, Kalunian K, Maddison P, Nived O, Petri M, Richter M, Sanchez-Guerrero J, Snaith M, Sturfelt G, Symmons D, Zoma A. The development and initial validation of the Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index for systemic lupus erythematosus. *Arthritis Rheum* 1996; **39**: 363-369
17 **Karhunen PJ**. Benign hepatic tumours and tumour like conditions in men. *J Clin Pathol* 1986; **39**: 183-188
18 **González Folch M**. [Erythema nodosum (author's transl)]. *Rev Med Chil* 1978; **106**: 915-922
19 **Glinkova V**, Shevah O, Boaz M, Levine A, Shirin H. Hepatic haemangiomas: possible association with female sex hormones. *Gut* 2004; **53**: 1352-1355
20 **Schnaper HW**, McGowan KA, Kim-Schulze S, Cid MC. Oestrogen and endothelial cell angiogenic activity. *Clin Exp Pharmacol Physiol* 1996; **23**: 247-250
21 **Folomeev M**, Dougados M, Beaune J, Kouyoumdjian JC, Nahoul K, Amor B, Alekberova Z. Plasma sex hormones and aromatase activity in tissues of patients with systemic lupus erythematosus. *Lupus* 1992; **1**: 191-195
22 **Robak E**, Woźniacka A, Sysa-Jedrzejowska A, Stepień H, Robak T. Serum levels of angiogenic cytokines in systemic lupus erythematosus and their correlation with disease activity. *Eur Cytokine Netw* 2001; **12**: 445-452
23 **Robak E**, Woźniacka A, Sysa-Jedrzejowska A, Stepień H, Robak T. Circulating angiogenesis inhibitor endostatin and positive endothelial growth regulators in patients with systemic lupus erythematosus. *Lupus* 2002; **11**: 348-355
24 **Wanless IR**, Mawdsley C, Adams R. On the pathogenesis of focal nodular hyperplasia of the liver. *Hepatology* 1985; **5**: 1194-1200
25 **Mathieu D**, Zafrani ES, Anglade MC, Dhumeaux D. Association of focal nodular hyperplasia and hepatic hemangioma. *Gastroenterology* 1989; **97**: 154-157
26 **Vilgrain V**, Uzan F, Brancatelli G, Federle MP, Zappa M, Menu Y. Prevalence of hepatic hemangioma in patients with focal nodular hyperplasia: MR imaging analysis. *Radiology* 2003; **229**: 75-79
27 **Bralet MP**, Terris B, Vilgrain V, Brégeaud L, Molas G, Corbic M, Belghiti J, Fléjou JF, Degott C. Epithelioid hemangioendothelioma, multiple focal nodular hyperplasias, and cavernous hemangiomas of the liver. *Arch Pathol Lab Med* 1999; **123**: 846-849
28 **Denninger MH**, Chaït Y, Casadevall N, Hillaire S, Guillin MC, Bezeaud A, Erlinger S, Briere J, Valla D. Cause of portal or hepatic venous thrombosis in adults: the role of multiple concurrent factors. *Hepatology* 2000; **31**: 587-591
29 **Caseiro-Alves F**, Brito J, Araujo AE, Belo-Soares P, Rodrigues H, Cipriano A, Sousa D, Mathieu D. Liver haemangioma: common and uncommon findings and how to improve the differential diagnosis. *Eur Radiol* 2007; **17**: 1544-1554

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BRIEF ARTICLE

Helicobacter pylori infection in bleeding peptic ulcer patients after non-steroidal antiinflammatory drug consumption

Francesco Manguso, Elisabetta Riccio, Germana de Nucci, Maria Luisa Aiezza, Gerardino Amato, Linda Degl'Innocenti, Maria Maddalena Piccirillo, Gianfranco De Dominicis, Tara Santoro, Elena Trimarco, Antonio Balzano

Francesco Manguso, Elisabetta Riccio, Germana de Nucci, Tara Santoro, Antonio Balzano, Complex Operating Unit of Gastroenterology, AORN A Cardarelli, 80131 Napoli, Italy
Maria Luisa Aiezza, Elena Trimarco, Complex Operating Unit of Pharmacy, AORN A Cardarelli, 80131 Napoli, Italy
Gerardino Amato, Linda Degl'Innocenti, Maria Maddalena Piccirillo, Complex Operating Unit of Microbiology, AORN A Cardarelli, 80131 Napoli, Italy
Gianfranco De Dominicis, Complex Operating Unit of Pathology, AORN A Cardarelli, 80131 Napoli, Italy
Author contributions: Manguso F, Riccio E and Balzano A conceived the study and wrote the article; de Nucci G and Santoro T collected data and reviewed the final version; Aiezza ML and Trimarco E determined the pharmacological history, collected data, and reviewed the final version; Amato G, Degl' Innocenti L, and Piccirillo MM performed the serological and microbiological tests, and reviewed the final version; De Dominicis G performed the histological evaluation and reviewed the final version.
Correspondence to: Dr. Francesco Manguso, Complex Operating Unit of Gastroenterology, AORN A Cardarelli, Via A Cardarelli 9, 80131 Napoli, Italy. manguso@alice.it
Telephone: +39-81-7474034 Fax: +39-81-7473018
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Abstract

AIM: To establish the prevalence of *Helicobacter pylori* (*H. pylori*) infection in patients with a bleeding peptic ulcer after consumption of non-steroidal antiinflammatory drugs (NSAIDs).

METHODS: A very early upper endoscopy was performed to find the source of upper gastrointestinal bleeding and to take biopsy specimens for analysis of *H. pylori* infection by the rapid urease (CLO) test, histological examination, and bacterial culture. IgG anti-CagA were also sought. The gold standard for identifying *H. pylori* infection was positive culture of biopsy specimens or contemporary positivity of the CLO test and the presence of *H. pylori* on tissue sections.

RESULTS: Eighty patients, 61 males (76.3%), mean age 61.2 ± 15.9 years, were consecutively enrolled. Forty-seven (58.8%) patients occasionally consumed NSAIDs, while 33 (41.3%) were on chronic treatment with low-dose aspirin (LD ASA). Forty-four (55.0%) patients were considered infected by *H. pylori*. The infection rate was not different between patients who occasionally or chronically consumed NSAIDs. The culture of biopsy specimens had a sensitivity of 86.4% and a specificity of 100%; corresponding figures for histological analysis were 65.9% and 77.8%, for the CLO test were 68.2% and 75%, for the combined use of histology and the CLO test were 56.8% and 100%, and for IgG anti-CagA were 90% and 98%. The highest accuracy (92.5%) was obtained with the culture of biopsy specimens.

CONCLUSION: Patients with a bleeding peptic ulcer after NSAID/LD ASA consumption frequently have *H. pylori* infection. Biopsy specimen culture after an early upper gastrointestinal tract endoscopy seems the most efficient test to detect this infection.

Key words: *Helicobacter pylori*; *Helicobacter pylori* infection; Low-dose aspirin; Non-steroidal antiinflammatory drugs; Peptic ulcer hemorrhage; Endoscopy

Peer reviewer: Hanna Gregorek, Assistant Professor, PhD,

Department of Microbiology and Clinical Immunology, The Children's Memorial Health Institute, Al Dzieci Polskich 20, Warsaw 04-730, Poland

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INTRODUCTION

Acute upper gastrointestinal (GI) bleeding is a life-threatening emergency frequently observed in patients admitted to tertiary care hospitals, with peptic ulcer bleeding accounting for approximately 50% of cases[1]. A high number of these emergency admissions for upper GI bleeding are attributable to non-steroidal anti-inflammatory drug (NSAID) use, especially in older patients[2,3].

The fact that peptic ulcers can be effectively treated by acid suppression strongly suggests that it is largely a disease of acid hypersecretion[3-5], although there is much evidence for a role of *Helicobacter pylori* (*H. pylori*) infection[6], and NSAID-induced injury[3]. Published data about the combined role of *H. pylori* infection and NSAID use in patients with peptic ulcer bleeding are conflicting. *H. pylori* infection has been demonstrated in a variety of studies, with a considerable degree of consistency, to increase the risk of NSAID-related GI injury[7]. Moreover, a recent meta-analysis indicated that prophylactic *H. pylori* eradication may help to reduce the risk of both gastric and duodenal ulcers and their complications, including bleeding, in chronic users of non-steroidal antiinflammatory drugs (NSAIDs)[8]. Other studies on the interaction between *H. pylori* infection and low-dose aspirin (LD ASA) use in patients with a history of upper GI bleeding demonstrated the protective role of *H. pylori* eradication on rebleeding. In these patients, *H. pylori* eradication may allow the use of LD ASA instead of other antithrombotic drugs[9]. On the other hand, some studies evidenced a lower rate of *H. pylori* infection in patients with bleeding peptic ulcers than in patients with uncomplicated peptic disease. In particular, a negative interaction between *H. pylori* infection and NSAID use was postulated, indicating a lower risk of bleeding in ulcer patients taking NSAIDs[10].

H. pylori infection can be diagnosed by invasive techniques requiring endoscopy and biopsy (histological examination, culture, and rapid urease test) and by non-invasive tests (serology, urea breath test, detection of *H. pylori* antigen in stool specimen). With the exception of the culture test, a single test has not reached acceptable accuracy for the diagnosis of *H. pylori* infection[11]. The rapid urease test and histological examination may indicate the presence of any urease-producing or helix-shaped bacteria, respectively. Moreover, serological tests are markers of exposure to *H. pylori* but do not indicate whether active infection is ongoing[11,12]. The results of the urea breath test are influenced by factors related to the patient, the bacteria, and the test itself[13]. Rapid gastric emptying, contamination with oral commensals, achlorhydria, and gastric atrophy may cause false positive results, while false negative results can occur through suppression of urease activity if the breath test is performed too soon after antibiotic or acid suppression therapy. The detection of *H. pylori* antigen in stools has some limitations related to bowel movements: a short transit time could favor elimination of unaltered antigens, while constipation could lead to degradation of the antigens[13]. Finally, in patients with recent upper GI bleeding the diagnosis of *H. pylori* infection can be challenging[14,15]. In fact, the hemorrhage itself (given the pH buffering effect of blood in the GI tract), the use of proton pump inhibitors and antibiotics may influence the results of invasive and non-invasive tests for *H. pylori*[15-17].

Our study was planned to give further information about the prevalence of *H. pylori* infection in occasional and chronic NSAID/LD ASA users admitted for peptic ulcer bleeding and submitted to very early upper endoscopy with a concomitant search for *H. pylori*.

MATERIALS AND METHODS

Ethics

The study was approved by the Institutional Review Board of the A. Cardarelli Hospital of Naples, Italy, and was conducted in compliance with the Declaration of Helsinki (1964 and following amendments), current Good Clinical Practices and the applicable European and local regulatory requirements.

Study design

This was a single-center, observational, prospective, registered study (EMOFANS Study: ACTRN12607000521426) carried out in the A. Cardarelli Hospital, a high volume hospital dedicated to emergencies. This study was planned to obtain information about the prevalence of *H. pylori* infection in patients regularly or occasionally consuming NSAIDs/LD ASA who were admitted for peptic ulcer disease complicated by hemorrhage. Taking into account our previous data, we calculated that it would be possible to recruit a total of 80 consecutive patients with the characteristics required by the protocol within a period of 12 mo. The primary objective of the study was to establish the prevalence of *H. pylori* infection in patients consecutively admitted to the emergency unit with upper GI bleeding from complicated peptic ulcer, who had been on chronic treatment or occasionally consumed NSAIDs/LD ASA. A secondary objective was to compare the efficiency of invasive (culture of biopsy specimens, *H. pylori* on tissue sections, rapid urease test) and non-invasive (IgG anti-CagA) techniques for the detection of *H. pylori* infection.

Table 1 Forrest classification

1 Actively bleeding ulcer
1a: Spurting
1b: Oozing
2 Non-actively bleeding ulcer
2a: Non-bleeding visible vessel
2b: Ulcer with surface clot
2c: Ulcer with red or dark blue spots
3 Ulcer with clean base

Inclusion and exclusion criteria

We recruited patients who fulfilled the following criteria: (1) male or female patients, of any ethnic origin, 18 years or more of age, who provided written informed consent prior to any study-related procedures and who were, in the opinion of the investigator, able to understand and to follow the protocol and likely to comply with all the requirements of the study; (2) patients with peptic ulcer disease complicated by hemorrhage (hematemesis, melena, hematochezia, or with other clinical signs of blood loss, i.e., hemodynamic instability with hypotension and tachycardia) in the 72 h before admission; (3) patients on chronic treatment with NSAIDs/LD ASA or who had received occasional treatment with NSAIDs in the 30 d before admission. Treatment with LD ASA was defined as the continuous use of up to 300 mg of aspirin per day for prophylaxis against vascular occlusive diseases[18]; and (4) patients with an ulcer, defined as a lesion with loss of mucosal integrity and continuity of $\geqslant$ 5 mm with an apparent depth of $\geqslant$ 1 mm, as measured using gastric biopsy forceps as standard. The exclusion criteria were: (1) patients who had received treatment with antibiotics or proton pump inhibitors within the 4 wk prior to potential enrolment in order to avoid false negative *H. pylori* results; (2) a history of previous major upper GI surgery; (3) patients with upper GI neoplastic ulcer; and (4) patients already hospitalized for other reasons.

Data collection

The patients' details were collected in a database and included: (1) demographic data; (2) comorbidities according to the Charlson Comorbidity Index[19]; (3) clinical and biochemical parameters; (4) occasional or regular use of NSAIDs/LD ASA; (5) other treatments taken; (6) endoscopy findings; (7) histology findings; (8) serological findings; and (9) microbiology results. The American Society of Anesthesiology classification of physical status[20] was calculated for each patient at admission prior to the endoscopy. Lesions were localized and classified according to the Forrest classification (Table 1)[21], and the Rockall risk score was also calculated after endoscopy[22].

Helicobacter pylori detection

The study plan included very early upper endoscopy, defined as within 6 h of arrival at the hospital[23], or as soon as possible after hemodynamic stabilization, to evaluate the source of upper GI bleeding and to take biopsies in eligible subjects. Gastric biopsies were obtained during endoscopy after successful hemostasis if needed. Biopsy specimens were taken from the antrum and gastric body to search for *H. pylori,* according to guidelines[24], and from all suspicious lesions. In particular, two specimens from the antrum as well as two specimens from the gastric body were used to perform the rapid urea (CLO) test (GASTREX, Warsaw, Poland) with separate kits. Moreover, two biopsy specimens from the antrum as well as two specimens each from the anterior and posterior parts of the gastric body were cultured for *H. pylori.* Finally, two biopsy specimens from the antrum and two specimens from the body were taken to detect *H. pylori* infection on tissue sections after modified Giemsa staining[25]. At the same time a blood sample was collected and IgG antibodies against CagA protein (DIA.PRO, Diagnostic Bioprobes Srl, Milan, Italy) were analyzed by enzyme immunoassays. CagA antibody titers (> 5 U/mL) were classified as positive according to the manufacturer's instructions.

The CLO test was carried out at room temperature, with the sample examined at 24 h, and considered positive when the appropriate color change (yellow to red) occurred. With regards to culture of biopsy specimens, primary isolation was performed on commercial selective Pylori agar (BioMérieux, 43263, Marcy-L'Étoile, France). Following primary selective isolation, *H. pylori* strains were identified by usual phenotypic tests (Gram stain and by oxidase, catalase and urease tests). To overcome the instability of *H. pylori* in biopsy material during its transport from the collection site to the laboratory, which is a limiting factor for culture and susceptibility testing, the biopsy specimens for the bacterial culture were immediately placed in an appropriate transport medium (Portagerm-Pylori, BioMérieux, 42041, Marcy-L' Étoile, France). The biopsy histology was interpreted by a GI pathologist blind to the patient's information and the results of the other *H. pylori* tests.

The gold standard for identifying *H. pylori* infection was a positive culture of biopsy specimens or contemporary positivity for the CLO test and the presence of *H. pylori* on tissue sections, in accordance with current guidelines[26].

Statistical analysis

Continuous data are expressed as means and standard deviations and compared by an independent samples *t* test. Categorical variables were analyzed by Pearson's chi-square test or Fisher's exact test. All tests of significance were two-sided. A *P*-value less than 0.05 was considered statistically significant. The PASW (Predictive Analytics Software) Statistics for Windows (Release 18.0.0-Jul 30, 2009; SPSS Inc., Chicago, Ill., United States) was used for the statistical analyses. Sensitivity, specificity, positive and negative predictive values, and false positive and false negative rates were determined using StatsDirect statistical software (release 2.7.8, March 15, 2010). The diagnostic accuracy was calculated as follow: Overall Ac-

Table 2 Demographic, clinical and endoscopic characteristics of all patients with bleeding from peptic ulcers after consumption of nonsteroidal anti-inflammatory drugs, grouped according to whether they did or did not have *Helicobacter pylori* infection *n* (%)

	Patients (*n* = 80)	*Helicobacter pylori* negative (*n* = 36)	*Helicobacter pylori* positive (*n* = 44)	*P*-value
Males	61 (76.3)	28 (77.8)	33 (75.0)	0.771
Age (yr) (mean ± SD)	61.2 ± 15.9	60.6 ± 18.3	61.7 ± 13.9	0.767
Smoker				0.703
Non-smoker	43 (53.8)	21 (58.3)	22 (50.0)	
Current	26 (32.5)	10 (27.8)	16 (36.4)	
Ex-smoker	11 (13.8)	5 (13.9)	6 (13.6)	
Symptoms on presentation				0.721
Hematemesis	11 (13.8)	4 (11.1)	7 (15.9)	
Melena	58 (72.5)	26 (72.2)	32 (72.7)	
Hematemesis and melena	11 (13.8)	6 (16.7)	5 (11.4)	
Initial mean hemoglobin (g/dL)	9.2 ± 2.3	8.7 ± 2.2	9.6 ± 2.4	0.076
American Society of Anesthesiology class				0.600
1-2	62 (77.5)	26 (72.2)	36 (81.8)	
3	15 (18.8)	8 (22.2)	7 (15.9)	
4	3 (3.8)	2 (5.6)	1 (2.3)	
Complete Rockall score				0.183
0-2	39 (48.8)	19 (52.8)	20 (45.5)	
3-5	34 (42.5)	12 (33.3)	22 (50)	
6-8	7 (8.8)	5 (13.9)	2 (4.5)	
[1]Comorbidity	27 (33.8)	12 (33.3)	15 (34.1)	0.943
Occasional consumption of NSAIDs	47 (58.8)	19 (52.8)	28 (63.6)	0.326
Patients on chronic LD ASA	33 (41.3)	17 (47.2)	16 (36.4)	0.326
Other antiplatelet drugs	5 (6.3)	2 (5.6)	3 (6.8)	0.999
Anticoagulants	2 (2.5)	2 (5.6)	0 (0)	0.199
Other drugs	39 (48.8)	15 (41.7)	24 (54.5)	0.252
Locations of ulcers				0.515
Duodenum alone	41 (51.3)	21 (58.3)	20 (45.5)	
Stomach alone	29 (36.3)	11 (30.6)	18 (40.9)	
Stomach and duodenum	10 (12.5)	4 (11.1)	6 (13.6)	
Forrest				0.684
1a	2 (2.5)	1 (2.8)	1 (2.3)	
1b	12 (15.0)	6 (16.7)	6 (13.6)	
2a	4 (5.0)	1 (2.8)	3 (6.8)	
2b	3 (3.8)	1 (2.8)	2 (4.6)	
2c	16 (20.0)	10 (27.8)	6 (13.6)	
3	43 (53.8)	17 (47.2)	26 (59.1)	

NSAID: Nonsteroidal anti-inflammatory drug; LD ASA; Low dose aspirin. [1]According to the Charlson Comorbidity Index. Because of rounding, not all percentages total 100.

curacy = (True Positive + True Negative)/(True Positive + False Positive + False Negative + True Negative).

RESULTS

Study population

Eighty consecutive patients (61 male, 19 female; mean age 61.2 ± 15.9 years (range, 21-85)) with upper GI bleeding from complicated peptic ulcer disease and on treatment with NSAIDs/LD ASA before admission, were enrolled. All patients were admitted to the emergency unit of the A. Cardarelli Hospital of Naples between January and December 2008. The characteristics of the study population are summarized in Table 2. No patients had a history of *H. pylori* eradication. In 67 (83.8%) patients endoscopic examinations took place within 6 h of arrival at the hospital, while in the remaining subjects endoscopies were delayed because of the patients' condition and were performed within 24 h. The site of the ulcers was duodenal in 41 patients (51.3%), gastric in 29 (36.3%), and in both segments in the remaining 10 patients (12.5%). In 14 cases (17.5%) the ulcers were classified as F1, in 23 (28.8%) as F2, and in 43 (53.8%) as F3 (Table 2). Six (7.5%) patients suffered rebleeding. None required surgery for bleeding or died during hospitalization. All patients were given proton-pump inhibitors intravenously in the emergency area and orally thereafter.

Consumption of non-steroidal antiinflammatory drugs

Most of the patients had occasionally consumed NSAIDs, particularly for a fever or moderate pain. In detail, 36 patients (45.0%) had occasionally consumed only one NSAID before their bleeding event, with one of these on concomitant chronic treatment with an antiaggregant (ticlopidine). Nine (11.3%) and two (2.5%) patients had consumed two and three NSAIDs in sequence, respectively. Thirty three patients (41.3%) were on chronic treatment with LD ASA for primary or secondary prevention of cardiovascular diseases., 18

Table 3 Patients' distribution on the basis of occasional or chronic consumption of nonsteroidal anti-inflammatory drugs

Occasional NSAID use	
One NSAID	35 (43.8)
Two NSAIDs (in sequence)	9 (11.3)
Three NSAIDs (in sequence)	2 (2.5)
NSAID + ticlopidine	1 (1.3)
Chronic NSAID use	
LD ASA alone	18 (22.5)
LD ASA + another NSAID[1]	8 (10.0)
LD ASA + two other NSAIDs (in sequence)[1]	1 (1.3)
LD ASA + ticlopidine	3 (3.8)
LD ASA + clopidogrel + NSAID[1]	1 (1.3)
LD ASA + LMWH	1 (1.3)
LD ASA + LMWH + 2 NSAIDs (in sequence)[1]	1 (1.3)

Data are expressed as number (percentage). NSAID: Non-steroidal anti-inflammatory drug; LD ASA: Low dose aspirin; LMWH: Low-molecular-weight heparin. [1]NSAIDs consumed occasionally. Because of rounding, not all percentages total 100.

Table 4 Frequency of positive diagnostic tests for *Helicobacter pylori* in culture-positive or culture-negative patients

	Culture-positive (n = 38)	Culture-negative (n = 42)
CLO test + tissue section-positive	19 (23.8)	6 (7.5)
CLO test positive	5 (6.3)	9 (11.3)
Tissue section-positive	4 (5.0)	8 (10.0)

Data are expressed as number (percentage).

(22.5%) on treatment with LD ASA alone, eight (10.0%) had occasionally consumed another NSAID, and one (1.3%) had taken two other NSAIDs in sequence. Four (5.0%) patients were on treatment with ticlopidine or clopidogrel (one of whom had occasionally consumed a NSAID), and two (2.5%) were receiving low-molecular-weight heparin (one of whom had consumed two NSAIDs in sequence) (Table 3). Thirty-nine (48.8%) patients were on treatment with other drugs considered not harmful to the intestinal mucosa. Twenty-eight out of 47 (59.6%) patients who occasionally consumed NSAIDs and 16/33 (48.5%) on chronic treatment with LD ASA were considered infected by *H. pylori*, with no statistically significant difference between the two groups (P = 0.326).

Diagnostic tests for Helicobacter pylori

Among the 80 bleeding patients, 38 (47.5%) had positive cultures of biopsy specimens, 37 (46.3%) had positive histopathological findings, 39 (48.8%) had a positive CLO test, and 36 (45.0%) were positive for IgG anti-CagA. The frequency of positive diagnostic tests for *H. pylori* in culture-positive or culture-negative patients is summarized in Table 4. Contemporaneous positivity of the CLO test and the presence of *H. pylori* on tissue sections were found in 25 (31.3%) patients. In particular, among 42 patients who had a negative culture of biopsy specimens, 6 (14.3%) had contemporary positivity of the CLO test and the presence of *H. pylori* on tissue sections. On the other hand, among 55 patients who did not have contemporary positivity of the CLO test and the presence of *H. pylori* on tissue sections, 19 (34.5%) had a positive culture of biopsy specimens. In accordance with the pre-established gold standard, 44 (55.0%) patients were considered infected by *H. pylori*. Among 44 infected, 25 (56.8%) had contemporary positivity of the CLO test and the presence of *H. pylori* on tissue sections, and 38 (86.4%) had a positive culture of biopsy specimens. With regards to IgG anti-CagA, among the infected and non-infected patients 26 (59.1%) and 10 (27.8%), respectively, had antibody titers > 5 U/mL.

Sensitivity, specificity, positive and negative predictive values, false positive rate, false negative rate and accuracy for all techniques are shown in Table 5. Culture of biopsy specimens had a sensitivity of 86.4% and a specificity of 100%. In this analysis, the sensitivities and specificities of the remaining tests were 65.9% and 77.8%, respectively, for histological analysis; 68.2% and 75.0%, respectively, for the CLO test; 56.8% and 100%, respectively, for the combined use of histology and the CLO test; and 90.0% and 98.0%, respectively, for the anti-CagA test. The highest accuracy (92.5%) was obtained with the culture of biopsy specimens.

The 80 patients with bleeding ulcers were divided into two groups for further analysis on the basis of presence or absence of *H. pylori* infection. These two groups were identical with regards demographic, clinical and endoscopic parameters (Table 2).

DISCUSSION

The reported prevalence of *H. pylori* infection in healthy persons (without GI illness) among many studies ranges from a minimum of 11% to a maximum of 69%, with some of the variability depending on the socioeconomic status of the country of the patients investigated[27,28]. The prevalence of *H. pylori* infection in patients with peptic ulcer disease is not well established as yet, especially because the prevalence of non-NSAID non-*H. pylori* ulcer is rising in the West, with current good evidence that 20%-40% of peptic ulcers are not associated with *H. pylori* infection or the use of NSAIDs[29]. In one study it was found that, after excluding NSAID users, only 61% of patients with peptic ulcers had *H. pylori* infection[30]. Data about *H. pylori* infection in patients with peptic ulcer disease complicated by hemorrhage, chronically or occasionally treated with NSAIDs/LD ASA are scarce. Such data might be difficult to collect, especially because of the choice of appropriate tests to detect *H. pylori* infection, and the timing of their performance in patients who bleed.

In our study *H. pylori* infection was found in 55% of patients with peptic ulcer disease complicated by hemorrhage after consumption of NSAIDs/LD ASA, who were not on treatment with antibiotics or proton-pump inhibitors. The Italian National Project for Gastrointestinal Bleeding (PNED) study reported a prevalence of *H. pylori* infection of 44.3% among patients who had bleeding from a non-variceal upper GI source, when the

Table 5 Performance of tests for *Helicobacter pylori* infection

	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	Accuracy (%)	False positive (%)	False negative (%)
Culture of biopsy specimens	38/44	36/36	38/38	36/42	74/80	0/36	6/44
	86.4 (72.7-94.8)	100 (90.3-100)	100 (90.8-100)	85.7 (71.5-94.6)	-92.5	0	-13.6
Helicobacter pylori on tissue	29/44	28/36	29/37	28/43	57/80	8/36	15/44
sections	65.9 (50.1-79.5)	77.8 (60.9-89.9)	78.4 (61.8-90.2)	65.1 (49.1-79.0)	-71.3	-22.2	-34.1
Rapid urease test	30/44	27/36	30/39	27/41	57/80	9/36	14/44
	68.2 (52.4-81.4)	75.0 (57.8-87.9)	76.9 (60.7-88.9)	65.9 (49.4-79.9)	-71.3	-25	-31.8
Helicobacter pylori on tissue	25/44	36/36	25/25	36/55	61/80	0/36	19/44
sections and rapid urease test	56.8 (41.0-71.7)	100 (90.3-100)	100 (86.3-100)	65.5 (51.4-77.8)	-76.3	0	-43.2
Anti-CagA	26/44	26/36	26/36	26/44	52/80	10/26	18/44
	59.1 (43.3-73.7)	72.2 (54.8-85.8)	72.2 (54.8-85.8)	59.1 (43.3-73.7)	-65	-38.5	-40.9

CI: Confidence interval; NPV: Negative predictive value; PPV: Positive predictive value.

presence of the infection was determined by histological evaluation performed during the patients' stay in hospital; 36% of the patients were on treatment with NSAIDs[31]. In our study, 46.3% of patients had positive histopathological findings, confirming the results of the PNED study[31]. Another study performed in patients who bled from peptic ulcers (57.4% users of NSAID and/or antiplatelet drugs) found, by histological examination and the CLO test, an overall prevalence of *H. pylori* infection of 53.7%. In detail, the prevalences according to the histological examination and the CLO test were 42.3% and 44.8%, respectively[32]. In a study by Schilling *et al* the CLO test was positive in 50% of patients, while *H. pylori* infection was detected by the ^{13}C-urea breath test and histological examination (gold standard) in 62% of the cases[33]. Our study showed positive CLO test results in 48.8% of cases.

Data about the use of culture of biopsy specimens for the detection of *H. pylori* in bleeding patients are scarce. Three studies performed between 1998 and 2000 involving a total of 314 patients showed percentages of *H. pylori*-positive patients from 24.7% to 69.1%[34-36]. In another study involving children with upper GI bleeding, *H. pylori* infection was considered to be present when histology and/or culture were positive; unfortunately, data about the culture test alone were not presented, but *H. pylori* infection was detected in 48.8% of patients, with 29.8% of the children on treatment with NSAIDs[37]. In our study 47.5% of patients had a positive culture of biopsy specimens.

We used a restrictive gold standard to consider a patient infected by *H. pylori*. In view of its absolute specificity, if culture alone was positive the patient was considered *H. pylori*-positive[26]. Moreover, because a patient with at least two positive tests should be considered as *H. pylori*-positive[26], we used as adjunctive gold standard, the contemporary positivity of the CLO test and the presence of *H. pylori* on tissue sections. In fact, the concomitant positivity of the rapid urease test and of the histological examination indicates, with the highest probability, the presence of helical urease-producing bacteria. Six patients whose cultures were negative showed contemporary positivity for the CLO test and the presence of *H. pylori* on tissue sections. This finding demonstrates that culture tests are responsible for a number, albeit low, of false negative results. On the other hand, by using the second gold standard, only 25 (31.3%) patients would have been considered infected by *H. pylori*, even though 37 (46.3%) had positive histopathological findings and 39 (48.8%) had a positive CLO test. Moreover, among 55 patients who did not have contemporary positivity of the CLO test and the presence of *H. pylori* on tissue sections, 19 had a positive culture of biopsy specimens. These results indicate that by using the criterion of at least two positive tests to consider patients as infected by *H. pylori*, many false negative results can be expected. In our study the combined use of three invasive tests performed during a very early upper endoscopy was adequate for the diagnosis of *H. pylori* infection. With regards to the non-invasive test, 36 (45%) patients were positive for IgG anti-CagA. This test showed a low sensitivity and specificity confirming that it is a rather inaccurate diagnostic method which cannot be recommended as the first diagnostic test for *H. pylori* infection.

Patients with complicated peptic ulcer disease are candidates for testing for *H. pylori* infection. Indeed, accurate and early diagnosis of *H. pylori* infection is a critical clinical problem in these patients. The discovery of the link between the *H. pylori* bacterium and peptic ulcer is one of the greatest breakthroughs in medical history, but it is surprising that so far a large bulk of data has led to discordant results in patients with hemorrhagic complicated disease, treated or not with NSAIDs. It has been suggested that the rate of *H. pylori* infection is lower in patients with bleeding peptic ulcer than in patients with uncomplicated peptic disease, and that there is a negative interaction between *H. pylori* infection and NSAID use[38,10]. Our study demonstrated that many patients with peptic ulcer disease complicated by hemorrhage and consuming NSAIDs/LD ASA are actually infected (55%), and that the infection may be detected with appropriate tests performed during a very early endoscopy. Culture of biopsy specimens appears to be more efficient than other techniques at detecting *H. pylori* infection (accuracy 92.5%). We believe that the discordant data in the literature are due to the different cohorts of

patients studied, the kinds of invasive/non-invasive test used, the timing at which the tests were performed, the contemporary use of proton-pump inhibitors or antibiotics, and resources available in the context in which the patient is admitted. We had two ideal conditions for eliminating some of these sources of variability: (1) the presence of a rota of gastroenterologists skilled in diagnostic and therapeutic measures available 24 h a day, 7 d a week (not as a 24-h "on call" service), and able to enroll all consecutive patients; and (2) close local collaboration between specialist gastroenterologists and microbiologists, with the possibility of methodologically sound performance of the culture tests, which is a tedious, time-consuming procedure that can be influenced by the transport conditions from the endoscopy room to the laboratory and the speed of processing, because the viability of the organism is reduced by exposure to atmospheric oxygen. Finally, we did not find a statistically significant difference in the percentage of *H. pylori* infections between patients who occasionally consumed NSAIDs and those on chronic treatment with LD ASA, indicating that chronic consumption does not modify the infection rate.

In conclusion, faced with a person with a bleeding peptic ulcer we suggest that invasive methods should be used to identify *H. pylori* infection. The accuracy of results of biopsy specimen culture in patients with peptic ulcer bleeding remains very high, and the sensitivity and specificity of this method do not seem to be affected by blood in the stomach or by the use of NSAIDs or LD ASA, when performed after a very early upper endoscopy.

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COMMENTS

Background

Helicobacter pylori (*H. pylori*) has been considered as a major cause of the development of peptic ulcer disease. Several studies have reported that the prevalence of *H. pylori* infection may be underestimated in patients with bleeding peptic ulcers. Moreover, knowledge regarding the detection of *H. pylori* infection in patients with peptic ulcer disease complicated by hemorrhage, chronically or occasionally treated with non-steroidal anti-inflammatory drugs (NSAIDs) is limited. Numerous invasive and non-invasive diagnostic methods are available for the detection of *H. pylori*. Effectiveness values of these tests may vary depending on the brand of test used, age of the population tested, treatment used and, probably, the bleeding situation.

Research frontiers

In this article, the authors assess the prevalence of *H. pylori* infection in patients with peptic ulcer disease complicated by hemorrhage after consumption of NSAIDs.

Innovations and breakthroughs

More than 50% of patients with peptic ulcer disease complicated by hemorrhage after consumption of NSAIDs are infected by *H. pylori*.

Applications

In these patients the authors recommend searching for *H. pylori* infection by using the culture of biopsy specimens after an early upper gastrointestinal tract endoscopy.

Peer review

This is an interesting and well presented study, in which the authors using an approach that overcomes the previous conditions negatively influencing results presented in the literature, clearly showed that invasive methods should be used to identify *H. pylori* infection in a patient with a bleeding peptic ulcer.

REFERENCES

1 **Fallah MA**, Prakash C, Edmundowicz S. Acute gastrointestinal bleeding. *Med Clin North Am* 2000; **84**: 1183-1208
2 **Van Dam J**, Brugge WR. Endoscopy of the upper gastrointestinal tract. *N Engl J Med* 1999; **341**: 1738-1748
3 **Chan FK**, Leung WK. Peptic-ulcer disease. *Lancet* 2002; **360**: 933-941
4 NIH Consensus Conference. Helicobacter pylori in peptic ulcer disease. NIH Consensus Development Panel on Helicobacter pylori in Peptic Ulcer Disease. *JAMA* 1994; **272**: 65-69
5 Current European concepts in the management of Helicobacter pylori infection. The Maastricht Consensus Report. European Helicobacter Pylori Study Group. *Gut* 1997; **41**: 8-13
6 **Konturek SJ**, Konturek PC, Konturek JW, Plonka M, Czesnikiewicz-Guzik M, Brzozowski T, Bielanski W. Helicobacter pylori and its involvement in gastritis and peptic ulcer formation. *J Physiol Pharmacol* 2006; **57** Suppl 3: 29-50
7 **Lanza FL**, Chan FK, Quigley EM. Guidelines for prevention of NSAID-related ulcer complications. *Am J Gastroenterol* 2009; **104**: 728-738
8 **Vergara M**, Catalán M, Gisbert JP, Calvet X. Meta-analysis: role of Helicobacter pylori eradication in the prevention of peptic ulcer in NSAID users. *Aliment Pharmacol Ther* 2005; **21**: 1411-1418
9 **Thiéfin G**. [Should Helicobacter pylori infection be tested and eradicated in patients treated or about to be treated with aspirin or nonsteroidal anti-inflammatory drugs?]. *Gastroenterol Clin Biol* 2003; **27**: 415-426
10 **Okan A**, Tankurt E, Aslan BU, Akpinar H, Simsek I, Gonen O. Relationship between non-steroidal anti-inflammatory drug use and Helicobacter pylori infection in bleeding or uncomplicated peptic ulcers: A case-control study. *J Gastroenterol Hepatol* 2003; **18**: 18-25
11 **Ricci C**, Holton J, Vaira D. Diagnosis of Helicobacter pylori: invasive and non-invasive tests. *Best Pract Res Clin Gastroenterol* 2007; **21**: 299-313
12 Guidelines for clinical trials in Helicobacter pylori infection. Working Party of the European Helicobacter pylori Study Group. *Gut* 1997; **41** Suppl 2: S1-S9
13 **Mégraud F**, Lehours P. Helicobacter pylori detection and antimicrobial susceptibility testing. *Clin Microbiol Rev* 2007; **20**: 280-322
14 **Chung IK**, Hong SJ, Kim EJ, Cho JY, Kim HS, Park SH, Lee MH, Kim SJ, Shim CS. What is the best method to diagnose Helicobacter infection in bleeding peptic ulcers?: a prospective trial. *Korean J Intern Med* 2001; **16**: 147-152
15 **Gisbert JP**, Abraira V. Accuracy of Helicobacter pylori diagnostic tests in patients with bleeding peptic ulcer: a systematic review and meta-analysis. *Am J Gastroenterol* 2006; **101**: 848-863
16 **Tang JH**, Liu NJ, Cheng HT, Lee CS, Chu YY, Sung KF, Lin CH, Tsou YK, Lien JM, Cheng CL. Endoscopic diagnosis of Helicobacter pylori infection by rapid urease test in bleeding peptic ulcers: a prospective case-control study. *J Clin Gastroenterol* 2009; **43**: 133-139
17 **Wildner-Christensen M**, Touborg Lassen A, Lindebjerg J, Schaffalitzky de Muckadell OB. Diagnosis of Helicobacter pylori in bleeding peptic ulcer patients, evaluation of urea-based tests. *Digestion* 2002; **66**: 9-13

18 **Lanas A**, Bajador E, Serrano P, Fuentes J, Carreño S, Guardia J, Sanz M, Montoro M, Sáinz R. Nitrovasodilators, low-dose aspirin, other nonsteroidal antiinflammatory drugs, and the risk of upper gastrointestinal bleeding. *N Engl J Med* 2000; **343**: 834-839

19 **Charlson ME**, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. *J Chronic Dis* 1987; **40**: 373-383

20 **Dripps RD**, LAMONT A, ECKENHOFF JE. The role of anesthesia in surgical mortality. *JAMA* 1961; **178**: 261-266

21 **Forrest JA**, Finlayson ND, Shearman DJ. Endoscopy in gastrointestinal bleeding. *Lancet* 1974; **2**: 394-397

22 **Rockall TA**, Logan RF, Devlin HB, Northfield TC. Risk assessment after acute upper gastrointestinal haemorrhage. *Gut* 1996; **38**: 316-321

23 **Barkun AN**, Bardou M, Kuipers EJ, Sung J, Hunt RH, Martel M, Sinclair P. International consensus recommendations on the management of patients with nonvariceal upper gastrointestinal bleeding. *Ann Intern Med* 2010; **152**: 101-113

24 **Mégraud F**, Lehours P. Helicobacter pylori detection and antimicrobial susceptibility testing. *Clin Microbiol Rev* 2007; **20**: 280-322

25 **Gray SF**, Wyatt JI, Rathbone BJ. Simplified techniques for identifying Campylobacter pyloridis. *J Clin Pathol* 1986; **39**: 1279

26 Technical annex: tests used to assess Helicobacter pylori infection. Working Party of the European Helicobacter pylori Study Group. *Gut* 1997; **41** Suppl 2: S10-S18

27 **Thjodleifsson B**, Asbjörnsdottir H, Sigurjonsdottir RB, Gíslason D, Olafsson I, Cook E, Gíslason T, Jogi R, Janson C. Seroprevalence of Helicobacter pylori and cagA antibodies in Iceland, Estonia and Sweden. *Scand J Infect Dis* 2007; **39**: 683-689

28 **Bruce MG**, Maaroos HI. Epidemiology of Helicobacter pylori infection. *Helicobacter* 2008; **13** Suppl 1: 1-6

29 **Chow DK**, Sung JJ. Non-NSAID non-H. pylori ulcer disease. *Best Pract Res Clin Gastroenterol* 2009; **23**: 3-9

30 **Jyotheeswaran S**, Shah AN, Jin HO, Potter GD, Ona FV, Chey WY. Prevalence of Helicobacter pylori in peptic ulcer patients in greater Rochester, NY: is empirical triple therapy justified? *Am J Gastroenterol* 1998; **93**: 574-578

31 **Marmo R**, Koch M, Cipolletta L, Capurso L, Grossi E, Cestari R, Bianco MA, Pandolfo N, Dezi A, Casetti T, Lorenzini I, Germani U, Imperiali G, Stroppa I, Barberani F, Boschetto S, Gigliozzi A, Gatto G, Peri V, Buzzi A, Della Casa D, Di Cicco M, Proietti M, Aragona G, Giangregorio F, Allegretta L, Tronci S, Michetti P, Romagnoli P, Piubello W, Ferri B, Fornari F, Del Piano M, Pagliarulo M, Di Mitri R, Trallori G, Bagnoli S, Frosini G, Macchiarelli R, Sorrentini I, Pietrini L, De Stefano S, Ceglia T, Chiozzini G, Salvagnini M, Di Muzio D, Rotondano G. Predicting mortality in non-variceal upper gastrointestinal bleeders: validation of the Italian PNED Score and Prospective Comparison with the Rockall Score. *Am J Gastroenterol* 2010; **105**: 1284-1291

32 **Tang JH**, Liu NJ, Cheng HT, Lee CS, Chu YY, Sung KF, Lin CH, Tsou YK, Lien JM, Cheng CL. Endoscopic diagnosis of Helicobacter pylori infection by rapid urease test in bleeding peptic ulcers: a prospective case-control study. *J Clin Gastroenterol* 2009; **43**: 133-139

33 **Schilling D**, Demel A, Adamek HE, Nüsse T, Weidmann E, Riemann JF. A negative rapid urease test is unreliable for exclusion of Helicobacter pylori infection during acute phase of ulcer bleeding. A prospective case control study. *Dig Liver Dis* 2003; **35**: 217-221

34 **Romero Gómez M**, Vargas J, Utrilla D, Rufo MC, Otero MA, Chavez M, Larraona JL, Castilla L, Guerrero P, Grande L, Castro Fernández M. [Prospective study on the influence of gastroduodenal ulcer hemorrhage on the diagnostic methods in Helicobacter pylori infection]. *Gastroenterol Hepatol* 1998; **21**: 267-271

35 **Tu TC**, Lee CL, Wu CH, Chen TK, Chan CC, Huang SH, Lee MS SC. Comparison of invasive and noninvasive tests for detecting Helicobacter pylori infection in bleeding peptic ulcers. *Gastrointest Endosc* 1999; **49**: 302-306

36 **Nousbaum JB**, Hochain P, Kerjean A, Rudelli A, Lalaude O, Herman H, Czernichow P, Dupas JL, Amouretti M, Gouerou H, Colin R. [Hemorrhaging eso-gastro-duodenal ulcers: epidemiology and management. A multicenter prospective study]. *Ann Chir* 1999; **53**: 942-948

37 **Boukthir S**, Mazigh SM, Kalach N, Bouyahya O, Sammoud A. The effect of non-steroidal anti-inflammatory drugs and Helicobacter pylori infection on the gastric mucosa in children with upper gastrointestinal bleeding. *Pediatr Surg Int* 2010; **26**: 227-230

38 **Huang JQ**, Sridhar S, Hunt RH. Role of Helicobacter pylori infection and non-steroidal anti-inflammatory drugs in peptic-ulcer disease: a meta-analysis. *Lancet* 2002; **359**: 14-22

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BRIEF ARTICLE

Impact of liver steatosis on response to pegylated interferon therapy in patients with chronic hepatitis B

Fehmi Ateş, Mehmet Yalnız, Saadet Alan

Fehmi Ateş, School of Medicine, Department of Gastroenterology, Mersin University, 33070 Mersin, Turkey
Mehmet Yalnız, School of Medicine, Department of Gastroenterology, Firat University, 23119 Elazığ, Turkey
Saadet Alan, Department of Pathology, Malatya Government Hospital, 44300 Malatya, Turkey
Author contributions: Ateş F and Yalnız M designed this research; Alan S performed pathologic investigation; Ateş F, Yalnız M and Alan S wrote this article.
Correspondence to: Dr. Fehmi Ateş, School of Medicine, Department of Gastroenterology, Mersin University, 33070 Mersin, Turkey. drfehmiates@hotmail.com
Telephone: +90-533-5296453 Fax: +90-324-3374305
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Abstract

AIM: To evaluate the impact of liver steatosis upon response to given therapy in chronic hepatitis B (CHB) patients.

METHODS: 84 consecutive CHB patients treated with 48-wk PEGylated interferon (PEG-IFN) therapy were enrolled. Baseline characteristics and sustained viral response (SVR) to PEG-IFN therapy were evaluated.

RESULTS: Mean body mass index (BMI) was 27.36 ± 4.4 kg/m^2. Six (7.1%) had hypertension and three (3.5%) had diabetes mellitus. Steatosis was present in 22.6% (19/84) of liver biopsy samples. Age, BMI, and triglyceride levels of the patients with hepatic steatosis were significantly higher than those without hepatic steatosis ($P < 0.05$). SVR to PEG-IFN therapy was 21.4% (18/84). Sixteen of these 18 CHB patients with SVR (88.9%) did not have any histopathologically determined steatosis. On the other hand, only two of the 19 CHB patients with hepatic steatosis had SVR (10.5%). Although the SVR rate observed in patients without steatosis (16/65, 24.6%) was higher compared to those with steatosis (2/19, 10.5%), the difference was not statistically significant ($P > 0.05$).

CONCLUSION: Occurrence of hepatic steatosis is significantly high in CHB patients and this association leads to a trend of decreased, but statistically insignificant, SVR rates to PEG-IFN treatment.

Key words: Chronic hepatitis B; Hepatic steatosis; PEGylated interferon therapy

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INTRODUCTION

Fatty livers, defined by the accumulation of lipid droplets, mainly triglycerides, in hepatocytes, are vulnerable to factors associated with further hepatic injury by their increased sensitivity to oxidative stress and to cytokine-mediated hepatic damage. This alone may not only lead to chronic liver disease, but can also influence the progression of chronic liver diseases with different etiologies and the response to given therapy. Steatosis, together with obesity and type 2 diabetes mellitus (DM), is also a proposed risk factor for the development of hepatocellular carcinoma[1].

Steatosis has been observed in the majority of chronic alcoholics and is also a common histopathological feature of chronic hepatitis C (CHC) infection. In patients with CHC, steatosis of the liver, accompanied by metabolic

and viral factors, increases the severity of fibrosis and unfavorably influences the response to given therapy[2-4]. Steatosis, however, may co-exist with other chronic liver diseases, in addition to alcoholic liver diseases and hepatitis C, because of the increasing prevalence of obesity and metabolic syndrome.

The number of studies reporting co-existence of steatosis and chronic hepatitis B (CHB), a major cause of chronic liver disease worldwide, is increasing. The impact of superimposed non-alcoholic fatty liver disease in patients with CHB, however, is less clear. There are only a few studies on this topic[5,6]. The components of metabolic syndrome [obesity, hypertension (HTA), and dyslipidemia] are associated with the presence of nonalcoholic steatohepatitis in patients with CHB, and the presence of hepatic fibrosis seems to be associated with known host and viral factors, as well as the presence of abdominal obesity[7].

We aimed to determine the frequency and risk factors of liver steatosis in patients with CHB, and to investigate its correlation with the response to given PEGylated interferon (PEG-IFN) therapy.

MATERIALS AND METHODS

Patients

Twenty-one hepatitis B e antigen (HBeAg) (+) and 63 HBeAg (-) (n = 84) consecutive patients with CHB, who were diagnosed by liver biopsy, and received 48-wk PEG-IFN therapy were enrolled in the study between December 2006 and July 2009. Patients were given either PEG-IFN α-2a or 2b. Forty patients received PEG-IFN α-2a 180 μg sc and 44 patients received Peg-IFN α-2b 1.5 μg/kg sc once a week. Sixty of the patients were male (71.4%) and 24 were (28.6%) female, their mean age was 38.6 ± 10.9 years and their age range was 18-61 years.

Before inclusion, the patients were informed and their written consents were obtained. The study protocol was approved by the local Ethics Committee, and the study was performed in accordance with the ethical standards laid down in an appropriate version of the 1975 Declaration of Helsinki.

Inclusion criteria

In the serum samples of the patients enrolled to the study, the hepatitis B surface antigen (HBsAg) had to have been present for more than 6 mo and, within the last 6 mo, at least two different measurements must have shown an elevation of alanine aminotransferase (ALT) > ULN × 2. Using polymerase chain reaction (PCR), it was found that HBV DNA levels were > 10 000 copies/mL in cases with HBeAg (-) and > 100 000 copies/mL in cases with HBeAg (+). The liver biopsies of all patients were consistent with the diagnosis of CHB.

Exclusion criteria

Patients who met at least one of the following were excluded from the study: patients co-infected with other viruses, such as hepatitis A, C, D, E, Cytomegalovirus, Epstein-Barr virus and HIV; patients with toxic hepatitis; patients with another liver disease; alcohol consumers (more than 20 g per day); patients who were taking antiviral drugs or interferon before the biopsy.

Body mass index (BMI) was calculated by dividing the body weight (kg) by the square of height (m). Based on the BMI values, < 25 kg/m^2 was considered as normal, 25-30 kg/m^2 as overweighed, and > 30 kg/m^2 as obese.

Serum analyses

Fasting blood samples were obtained 1 d before the liver biopsy, and ALT, aspartate aminotransferase, γ glutamyltransferase, glucose (GLU), cholesterol, and triglyceride levels were measured.

Virological analyses

For the analyses, HbsAg, HBeAg, Anti-HBe, Anti-HBc ARCHITECT chemiluminescent microparticle immunoassay kits (Abbott Park, Wiesbaden-Delkenheim, Germany) and ARCHITECT i2000 system were used. HBV DNA levels were studied quantitatively using an HBV RG PCR Kit (sensitivity: 100 copies/mL) and Rotor-Gene 3000 (Corbett Research) device. Sustained viral response (SVR) was defined as the fall in HBV DNA to undeterminable levels (< 100 copies/mL) 6 mo after (week 72) the end of 1-year PEG-IFN α therapy and disappearance of HBeAg in cases with HBeAg (+).

Histological evaluation

All percutaneous liver biopsies were performed by two experienced gastroenterologists using a 16-gauge needle. All histological analyses were performed by one experienced pathologist who was blinded to the study. Necroinflammation was determined by scoring according to Knodell's histological activity index (HAI): portal inflammation (0-4), lobular degeneration and focal necrosis (0-4), periportal ± bridging necrosis (0-10)[8]. The stage of fibrosis was classified from "no fibrosis" (Stage 0) to cirrhosis (Stage 4). Grading of hepatosteatosis was semiquantitatively performed according to hepatocyte involvement: None:0, Mild: 0%-10%, Moderate:10%-30%, Marked: 30%-60%, and Severe: > 60%[9].

Statistical analysis

While the numerical data of the patients were presented as mean ± SD, categorical variables were presented together with frequency and percentages. The intergroup differences of numerical variables were investigated using Student's t test, while the differences of categorical values were investigated using the χ^2 test. Variables that were found to be significant in univariate analysis (P < 0.05) were subjected to a multivariate logistic regression model to be investigated. All analyses were performed using a statistical software program (SPSS version 15.0).

RESULTS

Patient characteristics

Among 84 patients enrolled to the study, six (7.1%) had HTA and three (3.5%) had DM. The mean BMI value was

Table 1 Demographic and clinical characteristics of the patients (mean ± SD)

Parameter	*n* (%)
Male	60 (71.4)
Female	24 (28.6)
Age (yr)	38.6 ± 10.9
HBeAg (+)	21 (25.0)
HBeAg (-)	63 (75.0)
Hypertension	6 (7.1)
Diabetes mellitus	3 (3.5)
BMI (kg/m^2)	27.4 ± 4.40
< 25	28 (33.3)
25-30	34 (40.5)
> 30	22 (26.2)
Glucose (mg/dL)	101.4 ± 26.0
Cholesterol (mg/dL)	182.7 ± 29.3
Triglyceride (mg/dL)	131.0 ± 55.4
AST (IU/L)	103.3 ± 39.5
ALT (IU/L)	136.8 ± 47.2
GGT (IU/L)	49.9 ± 41.1
ALP (IU/L)	91.9 ± 26.5
HBV-DNA (copies/mL × 10^4)	5502.9 ± 11889.7

HBeAg: Hepatitis B e antigen; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ glutamyl transferase; ALP: Alkaline phosphatase; HBV: Hepatitis B virus.

Table 2 Histological characteristics of the patients

Parameter	*n* (%)
HAI score	
0-3	5 (5.9)
4-8	56 (66.7)
9-12	22 (26.2)
13-18	1 (1.2)
Stage of fibrosis	
0	0 (0.0)
1	7 (8.3)
2	42 (50.0)
3	35 (41.7)
4	0 (0.0)
Steatosis	
None (0)	65 (77.4)
Mild (< 10%)	7 (8.3)
Moderate (10%-30%)	7 (8.3)
Marked (30%-60%)	4 (4.8)
Severe (> 60%)	1 (1.2)

HAI: Histological activity index.

27.36 ± 4.4 kg/m^2. When the patients were evaluated according to their BMI, 28 (33.3%) were grouped as normal (< 25 kg/m^2), 34 (40.5%) as overweighed (25-30 kg/m^2), and 22 (26.2%) as obese (> 30 kg/m^2). The demographic, clinical, and laboratory data of the patients are presented in Tables 1 and 2.

Incidence of liver steatosis and related factors

Hepatosteatosis was histologically present in 19 of 84 patients with CHB (22.6%). For patients with hepatosteatosis, 36.8% (7/19) showed mild, 36.8% (7/19) moderate, 21.1% (4/19) marked, and 5.3% (1/19) severe hepatosteatosis. The factors that were statistically correlated with hepatosteatosis were patient's age, BMI, and triglyceride (TG) levels. In the hepatosteatosis group, the age (50.5 ± 8.7 years *vs* 35.2 ± 8.9 years, $P < 0.01$), BMI (32.9 ± 3.1 kg/m^2 *vs* 25.7 ± 3.3 kg/m^2, $P < 0.01$), and TG levels (188.3 ± 52.0 mg/dL *vs* 114.2 ± 44.2 mg/dL, $P < 0.01$) were higher compared to the group without hepatosteatosis. No significant correlation was found between hepatosteatosis and other parameters, such as HBeAg status, stage of fibrosis, HAI score, or HBV DNA level ($P > 0.05$). In multivariate analysis, it was found that advanced age, increased BMI, and elevated TG are independent predictors of the presence of hepatosteatosis (Table 3).

Table 3 Comparison between patients with and without hepatosteatosis (mean ± SD)

Parameter	Steatosis (+) (*n* = 19)	Steatosis (-) (*n* = 65)	*P* value
Male	14 (73.07)	46 (70.8)	NS
Female	5 (26.3)	19 (29.2)	NS
Age (yr)	50.5 ± 8.7	35.2 ± 8.9	< 0.01
BMI (kg/m^2)	32.9 ± 3.1	25.7 ± 3.3	< 0.01
⩾ 25	18 (94.7)	38 (58.5)	< 0.01
Glucose (mg/dL)	102.7 ± 27.7	96.7 ± 19.0	NS
Cholesterol (mg/dL)	192.2 ± 28.0	178.0 ± 27.4	NS
Triglyceride (mg/dL)	188.3 ± 52.0	114.2 ± 44.2	< 0.01
AST (IU/L)	90.7 ± 34.8	107.0 ± 40.3	NS
ALT (IU/L)	128.3 ± 18.9	139.2 ± 52.5	< 0.01
GGT (IU/L)	49.8 ± 29.8	50.0 ± 44.0	NS
ALP (IU/L)	91.8 ± 26.3	91.9 ± 26.7	NS
HBV-DNA (copies/mL × 10^4)	5261.6 ± 2394.9	5684.2 ± 13245.8	NS
HBeAg (+)	4 (21.1)	17 (26.2)	NS
HBeAg (-)	15 (78.9)	48 (73.8)	NS
Advanced fibrosis (score ⩾ 3)	8 (42.1)	27 (41.5)	NS
Advanced HAI (score ⩾ 9)	6 (31.5)	17 (26.2)	NS
SVR	2 (10.5)	16 (24.6)	NS

BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ glutamyl transferase; ALP: Alkaline phosphatase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HAI: Histological activity index; SVR: Sustained viral response. NS: Non significant.

Factors associated with SVR

Among 84 patients with CHB who received PEG-IFN therapy, 21.4% (18/84) showed SVR. The rate of SVR was 23.8% (5/21) in cases with HBeAg (+) and 20.6% (13/63) in cases with HBeAg (-). 88.8% (16/18) of CHB patients with SVR did not have any histopathologically determined steatosis. On the other hand, only two of the 19 CHB patients with liver steatosis - one with mild steatosis and the other one with moderate steatosis - had an SVR (10.5%). Although the SVR rate observed in patients without hepatosteatosis (16/65, 24.6%) was higher compared to those with hepatosteatosis (2/19, 10.5%), the difference was not statistically significant ($P > 0.05$). Using multivariate analysis, it was found that only ALT elevation was a independent predictor of SVR. No significant difference was found between SVR (+) and SVR (-) groups in terms of other parameters (Table 4). There

Table 4 Comparison between patients with and without sustained viral response (mean ± SD)

Parameter	SVR(+) (n = 18)	SVR (-) (n = 66)	P value
Male	13 (72.2)	47 (71.2)	NS
Female	5 (27.8)	19 (28.8)	NS
Age (yr)	35.3 ± 8.0	39.7 ± 10.8	NS
BMI (kg/m^2)	26.5 ± 3.1	27.9 ± 4.5	NS
⩾ 25	11 (61.1)	45 (68.2)	NS
Glucose (mg/dL)	97.4 ± 28.0	102.4 ± 25.5	NS
Cholesterol (mg/dL)	162.1 ± 23.7	175.6 ± 30.2	NS
Triglyceride (mg/dL)	109.7 ± 50.8	136.8 ± 28.5	NS
AST (IU/L)	117.2 ± 55.4	97.8 ± 28.5	NS
ALT (IU/L)	199.8 ± 82.0	122.3 ± 49.8	< 0.01
GGT (IU/L)	62.9 ± 69.9	46.4 ± 28.6	NS
ALP (IU/L)	93.4 ± 38.8	91.5 ± 22.4	NS
HBV-DNA (copies/mL × 10^4)	4961.6 ± 2245.1	5779.5 ± 13129.5	NS
HBeAg (+)	5 (27.8)	16 (24.2)	NS
HBeAg (-)	13 (72.2)	50 (75.8)	NS
Advanced fibrosis (score ⩾ 3)	7 (38.9)	28 (42.4)	NS
Advanced HAI (score ⩾ 9)	5 (27.8)	18 (27.8)	NS
Hepatosteatosis	2 (11.1)	17 (25.8)	NS

BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ glutamyl transferase; ALP: Alkaline phosphatase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HAI: Histological activity index; SVR: Sustained viral response. NS: Non significant.

Table 5 Comparison between hepatitis B e antigen (+) patients with and without sustained viral response *n* (%)

	SVR (+) (n = 5)	SVR (-) (n = 16)	P value
Hepatosteatosis (+)	1 (20)	3 (23)	NS
Hepatosteatosis (-)	4 (80)	13 (77)	NS

SVR: Sustained viral response. NS: Non significant.

Table 6 Comparison between hepatitis B e antigen (-) patients with and without sustained viral response

	SVR (+) (n = 13)	SVR (-) (n = 50)	P value
Hepatosteatosis (+)	1 (7.7)	14 (7.0)	NS
Hepatosteatosis (-)	12 (92.3)	36 (93.0)	NS

SVR: Sustained viral response. NS: Non significant.

was no difference in patients with or without steatosis regarding treatment response between the types of PEG-interferon (data not shown).

Subgroup analysis according to the HBeAg status

In HBeAg (-) patients, SVR was seen 7.7% (1/13) patients with hepatosteatosis, while it was 92.3% (12/13) without hepatosteatosis. Although the SVR rate was higher in the patients without hepatosteatosis, the difference was not statistically significant (Table 5).

In HBeAg (+) patients, SVR was seen in 20% (1/5) patients with hepatosteatosis while it was 80% (4/5) patients without hepatosteatosis. This difference was also not statistically significant (Table 6).

HBeAg seroconversion was seen in only four (19%) patients; all four patients had undetectable HBV DNA levels. None of our patients had HBsAg seroconversion.

DISCUSSION

This study investigated the incidence and clinical importance of liver steatosis in patients with CHB. The factors associated with liver steatosis were determined. In patients who were given 48 wk PEG-IFN α therapy, the effects of liver steatosis and other factors upon persistent viral response were also investigated. In previous studies, the incidence of hepatosteatosis in patients with CHB was reported to range between 4.5% and 76%[9-14]. Especially in the studies where the alcohol consumers are not excluded, patients with CHB showed higher rates of hepatosteatosis[15,16]. In the present study, hepatosteatosis was histopathologically determined in 22.6% of CHB patients, and this prevalence is similar to that of the general population. Cases with other accompanying liver diseases, such as hepatitis C or alcohol consumers, were excluded and, thereby, misleading results were avoided.

In the development of non-alcoholic hepatosteatosis, insulin resistance constitutes the main mechanism[17]. Insulin resistance leads to hyperinsulinemia and an increase in free fatty acid concentrations, resulting with TG accumulation in hepatocytes[18]. Insulin might also play an important role in the development of fibrosis accompanied by hepatosteatosis by activating the profibrogenic pathways[19]. The cause and clinical importance of hepatosteatosis accompanying CHB are not well defined. In previous studies, non-alcoholic hepatosteatosis seen in patients with CHB was related to advanced age, large waist circumference, high fasting GLU and C-peptide levels, HTA, or dyslipidemia[10,20]. In the present study, we found that advanced age, higher BMI, and elevated TG levels were independent risk factors of hepatosteatosis in patients with CHB. Hepatosteatosis and fibrosis scores, however, were not correlated.

The virus by itself might be the cause of the hepatosteatosis as seen in some CHC patients with hepatic steatosis[21]. However, we did not find any correlation between hepatosteatosis and viral factors, such as HBeAg status, HBV DNA level, and HAI. Taken together, the presence of steatosis correlates with some host factors (advanced age, high BMI, and TG levels), but not with viral genotype or viral load. Accordingly, the results of the present study support the finding that metabolic factors, rather than viral factors, are more determinant for hepatosteatosis encountered in cases with CHB[22] and that, whereas the association between steatosis and HCV is specific, this not the case in HBV-infected patients.

Hepatosteatosis is related to metabolic factors, and hepatitis C virus infection *per se* leads to hepatosteatosis

directly in different genotypes (in genotype 2 and 3)[21]. The presence of liver steatosis in chronic viral hepatitis B might vary according to different genotypes, as reported in CHC[3,4,20]. In the present study, HBV genotyping could not be performed due to lack of laboratory resources. Nevertheless, hepatitis B infection in Turkey is accepted to be virtually all genotype D (almost 100%); hence, a genotype effect is not expected, and analyzing the genotype is not recommended as cost-effective in such studies.

Treatment of CHB is a big challenge. The response rates are still low despite novel therapy strategies. Besides the viral factors, other accompanying conditions might hamper the success of a therapy. Hepatosteatosis encountered in other chronic liver diseases not only has the potential to influence the progression of diseases, but is also suggested to diminish the response to the given therapy[23]. In the literature, there is only one study that retrospectively investigated the effect of co-existent steatosis upon the response to treatment in CHB patients[24]. That study reported that the presence of steatosis does not have any effect on the outcome of the treatment. In the present study, persistent viral response to 48 wk of PEG-IFN was 21.4%, which was consistent with previous studies[25,26]. As a support for the study of Moucari *et al*[25], only ALT elevation was an independent predictor of SVR. Strikingly, 88.9% (16/18) of CHB patients with SVR did not have any histopathologically determined steatosis. On the other hand, only two of the 19 (10.5%) CHB patients with liver steatosis had SVR. The high SVR rates obtained in patients without hepatosteatosis compared to those with hepatosteatosis, however, were not significant statistically. The fact that hepatosteatosis has no statistically significant effect on SVR may be due to our small number of patients.

It would be better if the homeostasis model assessment (HOMA) could also be determined. However, HOMA was designed to determine the relationship between chronic viral hepatitis and the presence of steatosis with respect to the effect upon treatment of viral hepatitis. The role of risk factors of steatosis, including the GLU HOMA index upon the course of chronic viral hepatitis B patients with steatosis, was beyond the scope of this study. This will be the subject of future studies.

In conclusion, hepatosteatosis is encountered frequently in patients with CHB. This association leads to a trend of decreased, but statistically insignificant, SVR rate to PEG-IFN treatment, both in HBeAg (+) and HBeAg (-) patients. Hepatic steatosis, a risk-free, benign condition in healthy subjects, might become a dangerous co-factor of disease progression when it is present in patients affected by another liver disease. It might affect the response to antiviral treatment and the significant negative effect of hepatosteatosis on response to therapy in CHB patients should be demonstrated using larger prospective studies. Advanced age, BMI, and high levels of TG are independent risk factors of hepatic steatosis development. Treatment strategies against obesity and TG elevations would have positive effects on CHB progression and the response to the given therapy. Hence, combating steatosis and its associated factors might aid in increasing the response to therapy in CHB patients.

COMMENTS

Background

Fatty livers encountered frequently in clinical practice may co-exist with other chronic liver diseases, and can influence the progression of the chronic liver diseases with different etiologies. The number of studies reporting co-existence of steatosis and chronic hepatitis B (CHB) is increasing. The impact of superimposed non-alcoholic fatty liver disease in patients with CHB, however, is less clear.

Research frontiers

Fatty livers are more vulnerable to factors associated with further hepatic injury because of their increased sensitivity to oxidative stress and cytokine-mediated hepatic damage, which may lead to chronic liver disease. The presence of a fatty liver can influence the progression of the chronic liver diseases with different etiologies and the response to given therapy. Steatosis of the liver in patients with chronic hepatitis C increases the severity of fibrosis and unfavorably influences the response given to therapy. Nevertheless, the association of liver steatosis and CHB, a major cause of chronic liver disease worldwide, is less clear.

Innovations and breakthroughs

Hepatosteatosis is not infrequent in patients with CHB. Advanced age, body mass index (BMI), and high levels of triglyceride (TG) are independent risk factors for the development of hepatic steatosis in patients with chronic viral hepatitis B. This coexistence leads to a trend of decreased, but statistically insignificant, sustained viral response rate to PEGylated interferon treatment both in hepatitis B e antigen (HBeAg) (+) and HBeAg (-) patients.

Applications

Hepatic steatosis, a risk-free, benign condition in healthy subjects, might become a dangerous co-factor of disease progression when it is present in patients affected by another liver disease. It might affect the response to antiviral treatment and the significant negative effect of hepatosteatosis upon response to therapy in CHB patients should be demonstrated using larger prospective studies. Given the importance of advanced age, BMI, and high levels of TG as being independent risk factors for development of hepatic steatosis, treatment strategies against obesity and TG elevations would have positive effects on CHB progression and the response to given the therapy. Hence, combating steatosis and its associated factors might aid in increasing the response to therapy in CHB patients.

Peer review

This study, even is observational, is fairly interesting.

REFERENCES

1 **Petta S**, Craxì A. Hepatocellular carcinoma and non-alcoholic fatty liver disease: from a clinical to a molecular association. *Curr Pharm Des* 2010; **16**: 741-752

2 **Westin J**, Nordlinder H, Lagging M, Norkrans G, Wejstål R. Steatosis accelerates fibrosis development over time in hepatitis C virus genotype 3 infected patients. *J Hepatol* 2002; **37**: 837-842

3 **Powell EE**, Jonsson JR, Clouston AD. Steatosis: co-factor in other liver diseases. *Hepatology* 2005; **42**: 5-13

4 **Castera L**, Chouteau P, Hezode C, Zafrani ES, Dhumeaux D, Pawlotsky JM. Hepatitis C virus-induced hepatocellular steatosis. *Am J Gastroenterol* 2005; **100**: 711-715

5 **Altlparmak E**, Koklu S, Yalinkilic M, Yuksel O, Cicek B, Kayacetin E, Sahin T. Viral and host causes of fatty liver in chronic hepatitis B. *World J Gastroenterol* 2005; **11**: 3056-3059

6 **Thomopoulos KC**, Arvaniti V, Tsamantas AC, Dimitropoulou D, Gogos CA, Siagris D, Theocharis GJ, Labropoulou-Karatza C. Prevalence of liver steatosis in patients with chronic hepatitis B: a study of associated factors and of relationship with fibrosis. *Eur J Gastroenterol Hepatol* 2006; **18**: 233-237

7 **Bondini S**, Kallman J, Wheeler A, Prakash S, Gramlich T, Jondle DM, Younossi ZM. Impact of non-alcoholic fatty liver disease on chronic hepatitis B. *Liver Int* 2007; **27**: 607-611
8 **Knodell RG**, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, Kiernan TW, Wollman J. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1981; **1**: 431-435
9 **Asselah T**, Boyer N, Guimont MC, Cazals-Hatem D, Tubach F, Nahon K, Daïkha H, Vidaud D, Martinot M, Vidaud M, Degott C, Valla D, Marcellin P. Liver fibrosis is not associated with steatosis but with necroinflammation in French patients with chronic hepatitis C. *Gut* 2003; **52**: 1638-1643
10 **Malhotra V**, Sakhuja P, Gondal R, Sarin SK, Siddhu M, Dutt N. Histological comparison of chronic hepatitis B and C in an Indian population. *Trop Gastroenterol* 2000; **21**: 20-21
11 **Czaja AJ**, Carpenter HA, Santrach PJ, Moore SB. Host- and disease-specific factors affecting steatosis in chronic hepatitis C. *J Hepatol* 1998; **29**: 198-206
12 **Czaja AJ**, Carpenter HA. Sensitivity, specificity, and predictability of biopsy interpretations in chronic hepatitis. *Gastroenterology* 1993; **105**: 1824-1832
13 **Bondini S**, Gramlich T, Ramsey L, Ong JP, Jondle DM, Boparai N, Gujral H, Younossi ZM. The impact of non-alcoholic fatty liver disease (NAFLD) on chronic hepatitis B. *Hepatology* 2006; **44** (Supp 1): 655A
14 **Tsochatzis E**, Papatheodoridis GV, Manesis EK, Chrysanthos N, Kafiri G, Archimandritis AJ. Hepatic steatosis in chronic hepatitis B (CHB) is due to host metabolic factors. *Hepatology* 2006; **44** (Supp 1): A652-A653
15 **Lefkowitch JH**, Schiff ER, Davis GL, Perrillo RP, Lindsay K, Bodenheimer HC, Balart LA, Ortego TJ, Payne J, Dienstag JL. Pathological diagnosis of chronic hepatitis C: a multicenter comparative study with chronic hepatitis B. The Hepatitis Interventional Therapy Group. *Gastroenterology* 1993; **104**: 595-603
16 **Gordon A**, McLean CA, Pedersen JS, Bailey MJ, Roberts SK. Hepatic steatosis in chronic hepatitis B and C: predictors, distribution and effect on fibrosis. *J Hepatol* 2005; **43**: 38-44
17 **Kahn SE**, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 2006; **444**: 840-846
18 **Meek SE**, Nair KS, Jensen MD. Insulin regulation of regional free fatty acid metabolism. *Diabetes* 1999; **48**: 10-14
19 **Hui JM**, Sud A, Farrell GC, Bandara P, Byth K, Kench JG, McCaughan GW, George J. Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression [corrected]. *Gastroenterology* 2003; **125**: 1695-1704
20 **Minakari M**, Molaei M, Shalmani HM, Alizadeh AH, Jazi AH, Naderi N, Shavakhi A, Mashayekhi R, Zali MR. Liver steatosis in patients with chronic hepatitis B infection: host and viral risk factors. *Eur J Gastroenterol Hepatol* 2009; **21**: 512-516
21 **Rubbia-Brandt L**, Leandro G, Spahr L, Giostra E, Quadri R, Malé PJ, Negro F. Liver steatosis in chronic hepatitis C: a morphological sign suggesting infection with HCV genotype 3. *Histopathology* 2001; **39**: 119-124
22 **Peng D**, Han Y, Ding H, Wei L. Hepatic steatosis in chronic hepatitis B patients is associated with metabolic factors more than viral factors. *J Gastroenterol Hepatol* 2008; **23**: 1082-1088
23 **Antúnez I**, Aponte N, Fernández-Carbia A, Rodríguez-Perez F, Toro DH. Steatosis as a predictive factor for treatment response in patients with chronic hepatitis C. *P R Health Sci J* 2004; **23**: 57-60
24 **Cindoruk M**, Karakan T, Unal S. Hepatic steatosis has no impact on the outcome of treatment in patients with chronic hepatitis B infection. *J Clin Gastroenterol* 2007; **41**: 513-517
25 **Moucari R**, Mackiewicz V, Lada O, Ripault MP, Castelnau C, Martinot-Peignoux M, Dauvergne A, Asselah T, Boyer N, Bedossa P, Valla D, Vidaud M, Nicolas-Chanoine MH, Marcellin P. Early serum HBsAg drop: a strong predictor of sustained virological response to pegylated interferon alfa-2a in HBeAg-negative patients. *Hepatology* 2009; **49**: 1151-1157
26 **Perrillo RP**. Therapy of hepatitis B -- viral suppression or eradication? *Hepatology* 2006; **43**: S182-S193

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BRIEF ARTICLE

YFa and analogs: Investigation of opioid receptors in smooth muscle contraction

Krishan Kumar, Ritika Goyal, Annu Mudgal, Anita Mohan, Santosh Pasha

Krishan Kumar, Ritika Goyal, Annu Mudgal, Santosh Pasha, Peptide Synthesis Laboratory, Institute of Genomics and Integrative Biology, Delhi 110007, India
Krishan Kumar, Anita Mohan, University School of Basic and Applied Sciences, GGSIP University, Sector-16 C, Dwarka, Delhi 110075, India
Krishan Kumar, Department of Chemistry, Motilal Nehru College, University of Delhi, Delhi 110021, India
Author contributions: Kumar K, Mohan A and Pasha S designed the study; Kumar K performed the majority of experiments; Goyal R, Kumar K and Pasha S analyzed the data; Kumar K wrote the first draft of manuscript; Goyal R and Mudgal A contributed to the final version of the manuscript.
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Correspondence to: Dr. Santosh Pasha, Scientist "G", Peptide Synthesis Laboratory, Institute of Genomics and Integrative Biology, Mall Road, Delhi 110007, India. spasha@igib.res.in
Telephone: +91-11-27667439 Fax: +91-11-27667471
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Abstract

AIM: To study the pharmacological profile and inhibition of smooth muscle contraction by YFa and its analogs in conjunction with their receptor selectivity.

METHODS: The effects of YFa and its analogs (D-Ala2) YFa, Y (D-Ala2) GFMKKKFMRF amide and Des-Phe-YGGFMKKKFMR amide in guinea pig ileum (GPI) and mouse vas deferens (MVD) motility were studied using an isolated tissue organ bath system, and morphine and DynA (1-13) served as controls. Acetylcholine was used for muscle stimulation. The observations were validated by specific antagonist pretreatment experiments using naloxonazine, naltrindole and norbinaltorphimine norBNI.

RESULTS: YFa did not demonstrate significant inhibition of GPI muscle contraction as compared with morphine (15% *vs* 62%, $P = 0.0002$), but moderate inhibition of MVD muscle contraction, indicating the role of κ opioid receptors in the contraction. A moderate inhibition of GPI muscles by (Des-Phe) YFa revealed the role of anti-opiate receptors in the smooth muscle contraction. (D-Ala-2) YFa showed significant inhibition of smooth muscle contraction, indicating the involvement of mainly δ receptors in MVD contraction. These results were supported by specific antagonist pretreatment assays.

CONCLUSION: YFa revealed its side-effect-free analgesic properties with regard to arrest of gastrointestinal transit. The study provides evidences for the involvement of κ and anti-opioid receptors in smooth muscle contraction.

Key words: Opioid receptor; Guinea pig ileum; Mouse vas deferens; Smooth muscle contraction; Gastrointestinal motility

Peer reviewer: Edward J Ciaccio, PhD, Research Scientist, Department of Medicine, HP 804, Columbia University, 180 Fort Washington Avenue, New York, NY 10032, United States

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INTRODUCTION

Centrally acting μ-opioid agonists are still the most widely used analgesics for the relief of severe pain, but their

utility is limited by a number of well-known side effects, including tolerance, physical dependence, respiratory depression, and adverse gastrointestinal effects. To rectify these complications, the effects of opioid drugs on gastrointestinal transit have been extensively studied using rat models. Transit arrest is a common effect of opioids in mammals but the underlying secretomotor changes appear to vary between species[1]. Studies on gut muscle contractility have demonstrated that the circular muscle contractility plays a dominant role in segmentation and peristaltic propulsion of the gut[2]. Also, the drug sensitivity of circular muscle contraction differs from that of longitudinal muscle contraction[3,4].

The efforts to study opioids and opioid-receptor pharmacology have largely relied upon the availability of highly subtype-selective agonists and antagonists. Although immunohistochemical studies have revealed that the opioid receptor subtypes, μ, δ and κ, are present in the neural tissue of the rat enteric nervous system (ENS), but not in smooth muscle cells[5,6], various other studies have indicated their involvement in intestinal smooth muscle movement. *In vivo* studies using the charcoal meal method have indicated that μ and δ receptor activation causes slow transit in rats, but κ receptor activation has negligible effect[7-9]. On the contrary, Mitolo-Chieppa *et al*[10] have reported the involvement of κ-opioid receptors in inhibiting gut motility. An *in vitro* study has indicated that activation of both μ and δ receptors has an inhibitory influence on the peristaltic reflex of the rat ileum[11]. Similarly, *in vitro* studies using electrical stimulation have indicated an inhibitory influence of δ receptors (but not of μ receptors) on longitudinal muscle contractions in the rat jejunum[12,13]. Thus, the ambiguity regarding the role of κ-opioid receptors in gastrointestinal and vas deferens motility still persists. Keeping in mind these findings and current efforts to develop peripherally acting opioid analgesics directed towards different opioid receptor profiles (e.g., δ agonists or mixed μ agonist/δ antagonists)[14,15], we designed the methionine-enkephalin-Arg6-Phe7 (MERF)-based chimeric opioid peptide analogs, which have an affinity for multiple opioid receptors, to study tolerance behavior and other side effects of opioids.

MERF peptide has overlapping sequences of Met-enkephalin and FMRF amide, belongs to the opioid family[16], and is comprehensively distributed in the central nervous system of different mammals[17]. Conversely, peptides of the NPFF [Neuropeptide FF (FLFQPQRFa)/FMRFa family antagonize morphine-induced supraspinal analgesia[18] and may function as endogenous anti-opioid agents[19]. NPFF has also been perceived to exhibit opioid effects along with a role in tolerance. The intriguing relationship between opioid and anti-opioid activity of the peptide can be attributed to the FMRF amino acid sequence at the C terminus of MERF. Along these lines, a chimeric peptide YFa (YGGFMKKKFMRF amide) of met-enkephalin and FMRFa was designed to determine the role of endogenous amphiactive sequences like MERF in analgesia, and its modulation[20]. YFa administered intraperitoneally induces naloxone-reversible antinociception, suggesting the involvement of opioid receptors in mediation of its antinociceptive effects. Moreover, YFa-potentiated morphine induced antinociception and attenuated the development of tolerance to morphine analgesia, suggesting its possible role in pain modulation[21]. mRNA expression studies have revealed that YFa produces κ receptor specific antinociception without any tolerance[22], and it further induced cross tolerance to 20 mg/kg morphine analgesia after 4 d pretreatment with 80 mg/kg YFa[23]. The results of these studies have been substantiated by forskolin-stimulated cAMP inhibition and Eu-GTP-γS binding studies[24].

In addition to YFa, its analogs (D-Ala2) YFa, Y (D-Ala2) GFMKKKFMRF amide, and Des-Phe (YGGFMKKKFMR amide) have also been studied. (D-Ala2) YFa (1 mg/mouse) administered intracerebroventricularlly (icv) with 5.86 nmol/L morphine (2 mg/mouse, icv) produced an additive antinociceptive effect, suggesting its modulatory role in opioid (morphine) analgesia[21]. Furthermore, mRNA studies have indicated that (D-Ala2) YFa acts mainly through δ receptors and partially through κ and μ opioid receptors[25], suggesting that D-Ala2 substitution in YFa leads to changes in its receptor selectivity from κ to δ subtype. Des-Phe (YGGFMKKKFMR amide) demonstrates the loss of mRNA expression of μ opioid receptor and shows κ opioid receptor agonist activity at a higher concentration (unpublished observations). Thus, the observed tolerance-free antinociception of YFa and its analogs prompted us to examine their other pharmacological properties so as to understand the role of opioid receptors in inhibition of gut motility and vas deferens contraction.

In our previous study, we observed early onset of antinociceptive effect (5 min) by chimeric peptide, YFa, which could be a result of direct opioid receptor stimulation and/or due to release of endogenous opioid peptides. In the present study, *in vitro* guinea pig ileum (GPI) and mouse vas deferens (MVD) assays were performed. These assays provided a more physiologically favorable environment for the ligand-receptor interaction to understand the peripheral action of the peptides, because these peripheral opioid responses are important for some of their therapeutic properties such as analgesia and side effects like constipation. The effect of opioid receptor activation in these isolated organ preparations is to reduce smooth muscle contraction *via* inhibition of excitatory neurotransmitter release, which is revealed by measuring the inhibitory action on electrically stimulated contraction of the ileal and vas deferens muscles.

MATERIALS AND METHODS

Peptide synthesis

Peptides YFa, (D-Ala2) YFa, (Des-Phe) YFa and Dynor-

phin A (Tyr1-Gly2-Gly3-Phe4-Leu5-Arg6-Arg7-Ile8-Arg9-Pro10-Lys11-Leu12-Lys13) [DynA(1–13)], were synthesized by the solid-phase method on an ACT-90 peptide synthesizer (Advanced ChemTech, Louisville, KY, United States) using the standard chemistry of 9-flourenylmethoxycarbonyl amino acids (Novabiochem, Laufelfigen, Switzerland) and 1-hydroxybenzotriazole/diisopropylcarbodiimide activation method on Rink amide-MBHA and Wang resin. The peptides were purified by RP-C18 column (mBondapak 10 mm, 7.8 mm × 300 mm; Waters, Milford, MA, United States) on semi-preparative reverse-phase HPLC (Waters 600) with a 40-min linear gradient from 10% to 90% acetonitrile (containing 0.05% trifluoroacetic acid) in water. The mass analysis of the peptides was done in linear positive ion mode by MALDI-TOF/TOF (Bruker Daltonics Flex Analysis, Germany) with 2, 5-dihydroxybenzoic acid as the matrix. The peptide sequence was confirmed by automated peptide sequencing (Procise 491; Applied Biosystems, Carlsbad, CA, United States).

Chemicals

All the chemicals including naloxonazine, naltrindole, norBNI and acetylcholine were purchased from Sigma (St. Louis, MO, United States). Morphine was obtained from AIIMS (New Delhi, India). All the peptides were dissolved in Mili-Q water.

Animals

Male guinea pigs, 300-400 g (AIIMS), were housed, two per cage, kept on a 12-h light/dark cycle, and fed standard rat chow and water *ad libitum*. Male albino mice were obtained from Maulana Azad Medical College (Delhi, India). Animals were housed in temperature-controlled room (25 ℃ ± 1 ℃) and exposed to a 12-h light/dark cycle. The animals were handled according to the guidelines of The Committee for the Purpose of Control and Supervision of Experiments on Animals, India, and the Animal Ethical Committee of the Institute of Genomics and Integrative Biology (Delhi, India).

Bioassay

The experimental procedures were essentially those used previously[26-28]. For GPI and MVD bioassay, tissue strips were obtained from adult male guinea pigs weighing 300-400 g and male Swiss albino mice weighing 25-30 g. All the animals were sacrificed by intraperitoneal administration of overdose thiopentone (200 mg/kg). Tissues were suspended under 1 g tension in a 10-mL organ bath chamber containing Tyrode solution at 37 ℃ and bubbled with 95% O_2 and 5% CO_2. The tissues were connected to an isotonic force transducer connected to eight channel organ baths (AD Instruments, Sydney, NSW, Australia) and allowed to equilibrate for 30-45 min. All the tissues were stimulated by chemical method using acetylcholine[29,30]. Only the tissue preparations that responded to 2×10^{-4} mol/L acetylcholine by producing contractions of more than 1.5 g tension, were used. Preparations were equilibrated for at least 1 h with washes every 10 min before exposure to drugs. At the start of each experiment, a maximum response to acetylcholine (10^{-6} mol/L) was obtained in each tissue to check its suitability and the responses to opioid antagonists were expressed as percentages of the maximum acetylcholine. Each experiment was repeated with at least four separate tissue preparations obtained from different animals. Naloxonazine and naltrindole, specific antagonists of μ and δ opioid receptors, were used as negative controls.

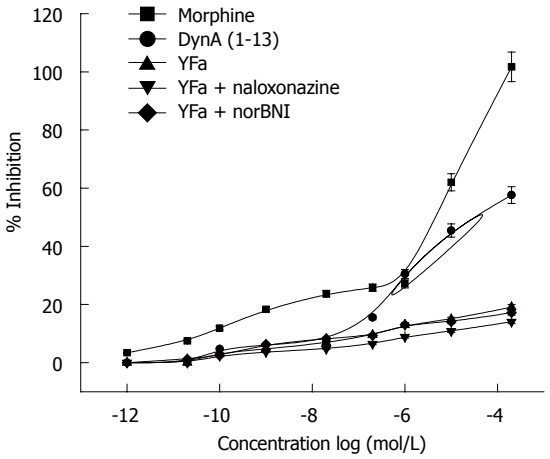

Figure 1 Guinea pig ileum assay of YFa. Morphine and DynA (1-13) were used as controls. Values represent mean ± SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and P = 0.0002.

Data analysis

GPI and MVD muscle contraction was measured as tension in grams. The inhibition percentage was calculated by taking acetylcholine contraction as 100% in all the tissues. All the assays were performed in triplicate and data were analyzed by Student's t test and one-way ANOVA in ORIGIN version 7.1. The data of each ligand were compared with morphine and DynA(1-13) separately, and $P < 0.05$ was considered statistically significant.

RESULTS

Effect of YFa on guinea pig ileum muscle contraction

In THE GPI assay (Figure 1), YFa demonstrated negligible inhibition of ileal muscle contraction, even at the highest concentration. Morphine, which interacts through μ opioid receptors, exhibited a highly significant inhibition rate of 62% (P = 0.001) at 10^{-5} mol/L and 101% at 2×10^{-4} mol/L. However, DynA (1-13), a known κ receptor agonist, showed a moderate inhibition of 57% (P = 0.004) at the highest dose of 2×10^{-4} mol/L.

Effect of YFa on mouse vas deferens muscle contraction

In MVD preparations (Figure 2), YFa exhibited a moderate inhibition of 24% (P = 0.001) at 2×10^{-7} mol/L and 45% at 10^{-5} mol/L. The maximum inhibitory response rate was 68% (P = 0.001), which was significantly lower

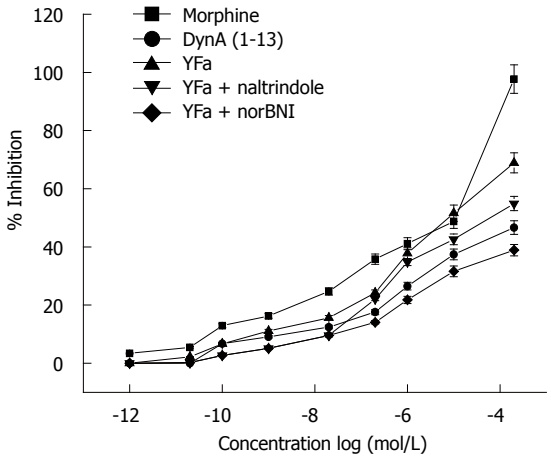

Figure 2 Mouse vas deferens assay of YFa. Morphine and DynA (1-13) were used as controls. Values represent mean ± SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and P = 0.001.

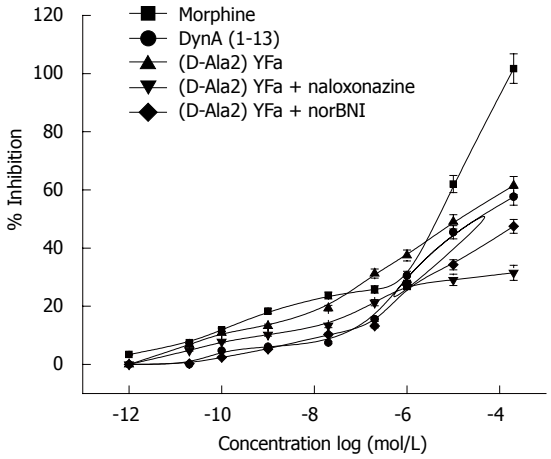

Figure 3 Guinea pig ileum assay of (D-Ala2) YFa. Morphine and DynA (1-13) were used as controls. Values represent mean ± SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and P = 0.0002.

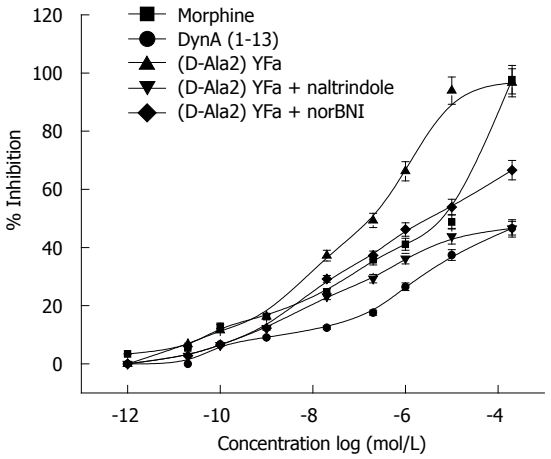

Figure 4 Mouse vas deferens assay of (D-Ala2) YFa. Morphine and DynA (1-13) were used as controls. Values represent mean ± SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and P = 0.0006.

Table 1 IC_{50} values for inhibition of smooth muscle contraction induced by YFa and its analogs in guinea pig ileum and mouse vas deferens assays

Guinea pig ileum (n = 9)[a]			Mouse vas deferens (n = 9)[a]		
Agonist	IC_{50} (μmol/L)	P value	Agonist	IC_{50} (μmol/L)	P value
Morphine	4.40	0.001	Morphine	13.41	0.001
Dyn A (1-13)	33.8	0.004	Dyn A (1-13)	ns	-
YFa	ns	-	YFa	7.10	0.001
D (Ala-2) YFa	12.0	0.001	D (Ala-2) YFa	0.20	0.0006
(Des-Phe) YFa	14.9	0.0002	(Des-Phe) YFa	ns	-
MERF-COOH	3.71	0.005	MERF-COOH	5.51	0.0005
MERF-NH2	ns	-	MERF-NH2	ns	-

[a]P < 0.05 for guinea pig ileum and mouse vas deferens assays was considered statistically significant. The values represent mean ± SE for three experiments performed in triplicate. IC_{50} values were calculated by one-way ANOVA and data were statistically significant. MERF: Methionine-enkephalin-Arg6-Phe7. ns: Non-significant.

than that of morphine (101%) but higher than DynA (1-13) (47%, P = 0.004). IC_{50} of YFa (7.10 μmol/L, P = 0.001) was nearly half that of morphine (13.41 μmol/L, P = 0.001), (Table 1). Vas deferens preparations pretreated with κ receptor specific antagonist norBNI showed a 44% reversibility of inhibitory activity, whereas, with naltrindole, the δ receptor specific antagonist, the activity was declined by only 20%.

Effect of (D-Ala2) YFa on guinea pig ileum muscle contraction

In contrast to YFa, (D-Ala2) YFa treatment resulted in moderate inhibition of GPI muscle contraction (Figure 3). It showed escalating behavior in inhibition from a value of 31.27 % (P = 0.0006) at 2 × 10^{-7} mol/L to 61.51% at 2 × 10^{-4} mol/L. The IC_{50} of (D-Ala2) YFa was 12 μmol/L (P = 0.001) (Table 1) compared with that of morphine (4.44 μmol/L, P = 0.001) (Table 1), which again indicated a moderate interaction with GPI muscle. Antagonist pretreatment of ileal tissue with naloxonazine exhibited a 50% decline in inhibition of muscle contraction, whereas only a 20% reversibility was observed with pretreatment with norBNI.

Effect of (D-Ala2) YFa on mouse vas deferens muscle contraction

(D-Ala2) YFa demonstrated a considerable inhibition of MVD muscle contraction (Figure 4), which increased progressively from 11.49% (P = 0.0006) at 10^{-10} mol/L to 37.22% at 2 × 10^{-8} mol/L (P = 0.0006). The maximum inhibitory response of 96% (P = 0.0006) at 2 × 10^{-4} mol/L was comparable to that of morphine (97%, P = 0.0006), but significantly higher than that of DynA (1-13) (46%). D-(Ala2) YFa showed an IC_{50} of 0.20 μmol/L (P = 0.0006) (Table 1), demonstrating the selective interaction of the peptide with δ opioid receptors, which are substantially present in MVD muscles.

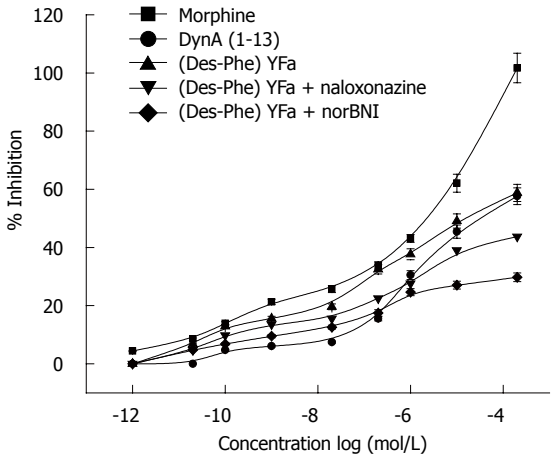

Figure 5 Guinea pig ileum assay of (Des-Phe) YFa. Morphine and DynA (1-13) were used as controls. Values represent mean ± SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and $P = 0.0002$.

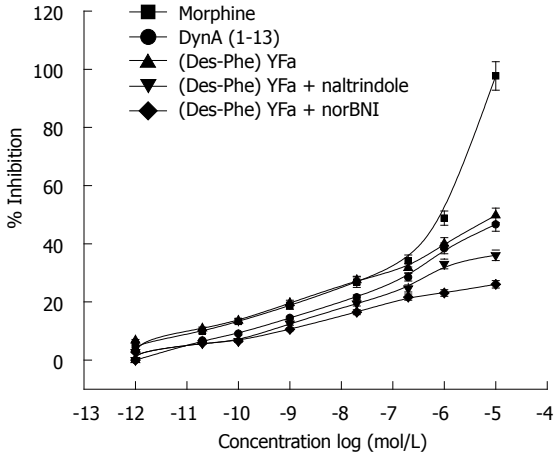

Figure 6 Mouse vas deferens assay of (Des-Phe) YFa. Morphine and DynA(1-13) were used as controls. Values represent mean ± SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and $P = 0.001$.

Pretreatment with naltrindole or norBNI resulted in a 52% ($P = 0.0006$) and 30% ($P = 0.0006$) reversibility of inhibition of MVD muscle contraction. DynA (1-13) also showed a weak inhibition of MVD contraction with a non-significant IC_{50} value.

Effect of (Des-Phe) YFa on guinea pig ileum muscle contraction

Moderately significant inhibition was observed with (Des-Phe) YFa treatment comparable to that of DynA(1-13) (Figure 5). The inhibitory response was stronger than that of YFa but weaker than that of morphine. A two-fold increase in inhibition from 15.54% ($P = 0.0002$) to 32.49% ($P = 0.0002$) was noted as concentration increased from 10^{-9} mol/L to 2×10^{-7} mol/L with IC_{50} at 14.9 μmol/L ($P = 0.0002$) (Table 1). The specific-antagonist-pretreated GPI preparations demonstrated that the reversibility in inhibitory activity of (Des-Phe) YFa was higher with norBNI (50%, $P = 0.0002$) than with naloxonazine (26%, $P = 0.0002$).

Effect of (Des-Phe) YFa on mouse vas deferens muscle contraction

(Des-Phe) YFa treatment resulted in a weak inhibition of MVD muscle contraction (Figure 6). The inhibitory response was comparable to that of dynorphin with a maximum response of 49.78% ($P = 0.001$) at 2×10^{-4} mol/L. Pretreatment with norBNI resulted in a 48% ($P = 0.001$) reversibility of inhibitory activity, while naltrindole pretreatment led to a 28% reversibility ($P = 0.001$).

Effect of MERF-COOH on guinea pig ileum muscle contraction

MERF-COOH, an endogenous opioid receptor agonist, has been reported to bind to all three subtypes of opioid receptors. GPI assay (Figure 7) showed a dose-dependent response that was comparable to that of morphine at all concentrations. Analogous to morphine and MERF-COOH, exhibited a steady rise in inhibition at a dose of 10^{-5} mol/L (58.57%, $P = 0.005$ and 62.10%, $P = 0.001$, respectively), and further demonstrated a sudden (almost twofold) elevation in inhibition profile by 95.27% ($P = 0.005$) at the highest dose. The IC_{50} value (3.71 μmol/L, $P = 0.005$) (Table 1) was comparable to that of morphine (4.40 μmol/L, $P = 0.001$). Moreover, a similar reversibility (50%) in inhibition profile was noted in the GPI preparations pretreated with norBNI and naloxonazine.

Effect of MERF-COOH on mouse vas deferens muscle contraction

In the MVD assay (Figure 8), MERF-COOH demonstrated a significant inhibition of MVD muscle contraction. At 10^{-6} mol/L concentration, it exhibited a comparable inhibition profile to morphine, whereas at higher concentrations, the trend varied. MERF-COOH exhibited an IC_{50} value of 5.51 μmol/L ($P = 0.001$), which was less than half that of morphine (13.41 μmol/L, $P = 0.001$) (Table 1). Moreover, the peptide showed a 72.57% ($P = 0.001$) inhibition at the highest dose. The δ- and κ-specific antagonist pretreatment of MVD preparations exhibited a similar degree of reversibility (35%) of inhibitory activity with naltrindole and norBNI, respectively.

Effect of MERF-NH_2 on guinea pig ileum and mouse vas deferens smooth muscle contraction

Contrary to MERF-COOH, MERF-NH_2 treatment resulted in a weak inhibition of GPI (41%, $P = 0.001$) and MVD (31%, $P = 0.0005$) muscle contraction at the highest concentration of 10^{-4} mol/L (Figures 9 and 10). The specific-antagonist-pretreated preparations of GPI (naloxonazine and norBNI) and MVD (naltrindole and norBNI) did not show any significant reversibility in inhibition profile.

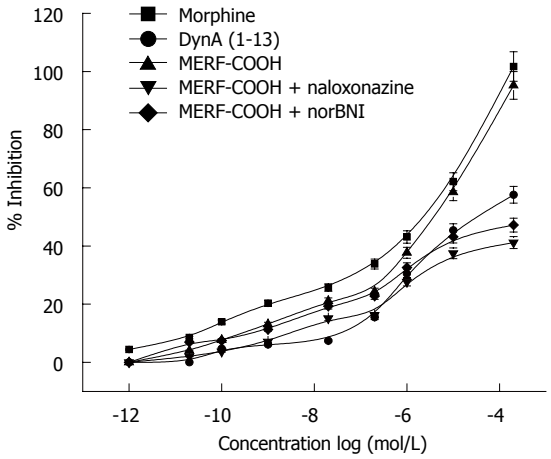

Figure 7 Guinea pig ileum assay of methionine-enkephalin-Arg6-Phe7-COOH. Morphine and DynA (1-13) were used as controls. Values represent mean ± SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and P = 0.005. MERF: Methionine-enkephalin-Arg6-Phe7.

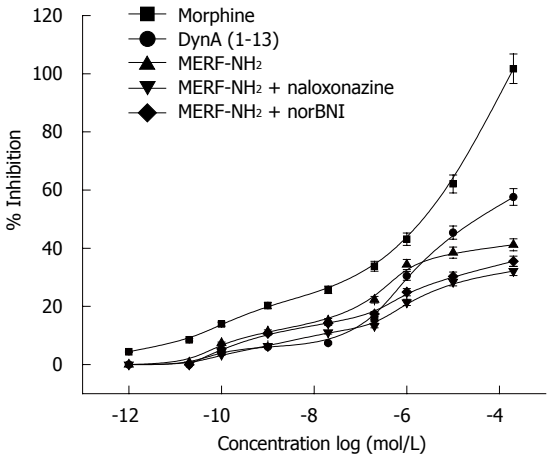

Figure 9 Guinea pig ileum assay of methionine-enkephalin-Arg6-Phe7-NH_2. Morphine and DynA (1-13) were used as controls. Values represent mean ± SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and P = 0.001. MERF: Methionine-enkephalin-Arg6-Phe7.

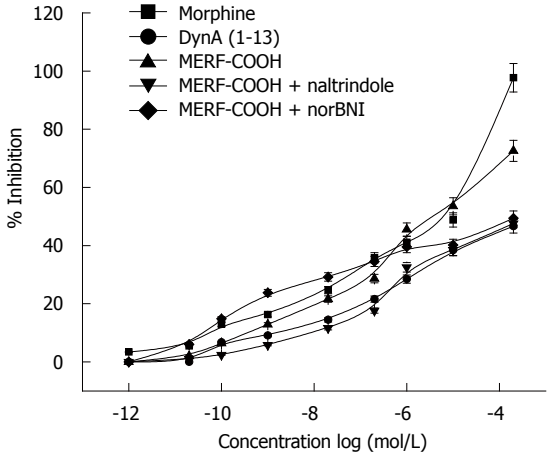

Figure 8 Mouse vas deferens assay of methionine-enkephalin-Arg6-Phe7-COOH. Morphine and DynA (1-13) were used as controls. Values represent mean ± SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and P = 0.001. MERF: Methionine-enkephalin-Arg6-Phe7.

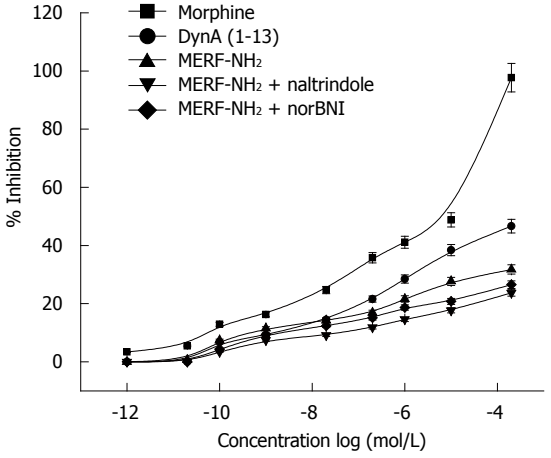

Figure 10 Mouse vas deferens assay of methionine-enkephalin-Arg6-Phe7-NH_2. Morphine and DynA (1-13) were used as controls. Values represent mean ± SE for three experiments performed in triplicate. Data were analyzed by one-way ANOVA using ORIGIN 7.1 and P = 0.0005. MERF: Methionine-enkephalin-Arg6-Phe7.

DISCUSSION

This study examines the effects of YFa and its analogs on GPI and MVD motility, in conjunction with their receptor selectivity. It is well documented that μ opioid receptors are primarily responsible for constipation, along with inhibition of nitric oxide generation[31]. In the gastrointestinal tract, activation of μ opioid receptors results in the inhibition of gut motility that leads to constipation, whereas similar receptors in the central nervous system mediate the analgesic actions of opioids[32]. The μ-receptor-selective drug, morphine, significantly restricted the smooth muscle contractions in lower intestine, indicating the presence of μ opioid receptors in the ileal muscle. Therefore, by inhibiting gastric flow and reducing propulsive peristalsis of the intestine, morphine decreases the rate of intestinal transit. Reduction in gut secretion and increase in intestinal fluid absorption further contribute to the constipating effect[33].

In 1993, Smith and Leslie[34] reported the δ subtype of opioid receptors as the major form in MVD, with a smaller number of μ receptors. Alternatively, in 1999, Pound[35] reported that morphine induced significant inhibition of MVD muscle contraction, which indicated the presence of separate μ opioid receptors. Furthermore, functional interactions between μ and δ opioid receptors, for several biochemical and pharmacological responses have also been reported by various groups[36-39]. These functional interactions of μ receptors could be rationalized on the basis of their indirect activation by δ receptors[40]. Collectively, these findings reveal that δ opioid receptors are prominent in MVD and there exists some cooperation between μ and δ opioid receptors that supports the hypothesis of synergistic interactions

between these two receptors.

Although the presence of μ receptors in the gut and MVD is well supported in the literature, the role of κ receptors is still ambiguous. Here, we used YFa and its analogs as probes to unravel these hidden aspects. Our previous studies on YFa have revealed its κ-receptor-selective nature. However, at higher concentrations, it also interacts with μ receptors[22-24]. In the present study, YFa showed a negligible inhibition of GPI contraction. This could be due to non-availability of κ opioid receptors or to the counteracting effect of the anti-opioid side (FMRF-amide) of the peptide, through its interaction with the anti-opiate receptors, by increasing sensitivity to cholinergic stimulation upon acetylcholine release[41-43].

To investigate further the reason behind these observations, an analog of YFa, (Des-Phe) YFa, was designed and studied. Upon modification, (Des-Phe) YFa retained its κ-receptor-selective antinociceptive nature but removal of Phe from the C terminus resulted in loss of RF-amide interaction with anti-opiate receptor, hence nullifying the counteractive effect of anti-opiate moiety (RFa) in YFa. (Des-Phe) YFa exhibited a significant inhibition of GPI muscle contractions, comparable to those of dynorphin. In comparison with YFa, a threefold increase in inhibition was observed as a result of the modification. Therefore, the counteractive effect of the anti-opioid side of YFa could be the reason for the non-significant inhibitory effect of YFa. This observation emphasizes the existence of κ-receptor-mediated contractions, in addition to the known μ receptor involvement in GPI muscle contractions[10,44-47]. Reversibility of contraction by pretreatment with κ-receptor-specific antagonist nor-BNI confirmed the κ-receptor-mediated interaction in GPI.

To substantiate the role of the anti-opiate moiety in the effect of YFa, analogs of MERF were studied. As mentioned earlier, MERF is a well-documented peptide belonging to the opioid family. Here, we studied the inhibitory profiles of two slight modifications of this peptide, MERF-COOH and MERF-NH_2, the latter of which has a C-terminal RFamide residue that interacts with the anti-opiate receptors. As expected, in the GPI assay, MERF-COOH led to a 100% inhibition and MERF-NH_2 treatment resulted in a negligible response. This complete reversal in properties confirms the role of counteractive effects of anti-opioid moieties in antinociception.

In the MVD assay, YFa demonstrated moderately significant inhibition of vas deferens contractions, in a dose-dependent fashion. This observation firmly suggests the involvement of the κ receptors in the observed effect, which corroborates the earlier reports suggesting the involvement of κ receptors in MVD muscle contraction[10,44,48]. Moreover, the maximum inhibitory response was found to be stronger than that of dynorphin (κ-receptor-specific agonist), suggesting the involvement of other receptors also, which may be due to the saturation of κ opioid receptors. The role of κ receptors was substantiated by antagonist pretreatment studies that showed a higher reversibility of contraction by κ-receptor than δ-receptor antagonist. The role of anti-opiate receptor is still not evident in MVD, therefore, that possibility was not considered in this case.

Recently, we have reported that (D-Ala2) YFa mediates its action primarily through δ opioid receptors and partially through μ and κ opioid receptors[25]. In the present study, (D-Ala2) YFa demonstrated a moderate inhibition of GPI muscle contraction, comparable to that of dynorphin, suggesting the involvement of μ and κ receptors in the observed effect. Specific antagonist pretreatment studies have emphasized the role of μ receptors and naloxonazine (μ-receptor antagonist) pretreatment resulted in a 50% reversibility in inhibition. The IC_{50} value of (D-Ala2) YFa of 12 μm, which was much lower than that of DynA (1-13) (33.8 μm), substantiated the role of μ receptors in (D-Ala2) YFa-mediated GPI contraction.

Furthermore, in the MVD assay, (D-Ala2) YFa demonstrated a significantly greater inhibition than that of morphine at all concentrations up to 10^{-5} mol/L. As expected, this suggests the involvement of δ and μ receptors in MVD muscle contraction. Pretreatment with naltrindole resulted in an almost 50% reduction in inhibition that suggested the involvement of δ receptors, which was further demonstrated by the IC_{50} value of (D-Ala2) YFa of 0.2 μmol/L ($P = 0.01$). However, significant inhibition of MVD muscle contraction by morphine (μ-receptor specific) and (D-Ala2) YFa (δ-receptor specific) further signifies that some cooperation may exist between μ and δ receptors in vas deferens preparations[40], or the δ receptors may regulate μ-receptor function *via* heterodimerization[49]. Further studies on heterodimerization of opioid receptors (μ, δ and κ) are required to elucidate their synergistic behavior and are currently in progress in our laboratory.

In conclusion, YFa and its analogs can be viewed as promising candidates to understand the role of opioid receptors in gastrointestinal transit and MVD motility. Although the precise mechanism by which anti-opiate receptors normalize the effects mediated by opioid receptors in GPI and MVD contraction is currently not clear, we provide convincing evidences that anti-opioid receptors are involved in the phenomenon. We also confirmed the presence of κ receptors in GPI and MVD muscles. Furthermore, the present findings provide a systematic approach to advance the researches on opioids due to the similar nature of opioid receptors in GPI and human intestines.

COMMENTS

Background

To date, centrally acting μ-receptor-specific agonists are the most widely used analgesics but their relieving effect is accompanied by a number of side effects including tolerance and adverse gastrointestinal effects.

Research frontiers

Opioids mediate their effects through various receptors (μ, κ and δ) present in the central nervous system, but the presence of similar receptors in the en-

teric nervous system leads to disturbances in gastrointestinal transit. Previous studies have reported the presence of μ and δ receptors in the gut and vas deferens, whereas the role of κ receptors is still ambiguous. In this study, the authors demonstrated the role of κ receptors and anti-opioid receptors using methionine-enkephalin-Arg6-Phe7 (MERF) peptide analogs.

Innovations and breakthroughs

The study reported YFa, an analgesic peptide molecule, free of gastrointestinal inhibition effect.

Applications

By understanding the roles of various opioid receptors in gastrointestinal transit, this study will provide a systematic approach to advance the researches on opioids.

Terminology

Guinea pig ileum (GPI) and mouse vas deferens (MVD) assays are the well reported methods for screening the drugs/molecules for smooth muscle contractions.

Peer review

Overall, the present work is a useful study which is potentially helpful for establishing the connection between opioid agents and smooth muscle contraction in humans. One goal is to develop pharmacological means to counteract undesirable effects of chronic administration of opioids in patients.

REFERENCES

1 **Miller RJ,** Hirning LD. Opioid peptides of the gut. Shultz SG, Wood JD, editors. Handbook of physiology-the gastrointestinal system II. Bethesda: American Physiological Society, 1989: 631-660
2 **Kosterlitz HW**, Lees GM. Pharmacological analysis of intrinsic intestinal reflexes. *Pharmacol Rev* 1964; **16**: 301-339
3 **Brownlee G**, Harry J. Some pharmacological properties of the circular and longitudinal muscle strips from the guinea-pig isolated ileum. *Br J Pharmacol Chemother* 1963; **21**: 544-554
4 **Coupar IM**. Characterization and tissue location of the neural adenosine receptor in the rat ileum. *Br J Pharmacol* 1999; **126**: 1269-1275
5 **Sternini C,** Spann M, De Giorgio R, Anton B, Keith D, Evans C, Brecha NC. Cellular localization of the μ-opioid receptor in the rat and guinea pig enteric nervous system. *Analgesia* 1995; **1**: 762-765
6 **Bagnol D**, Mansour A, Akil H, Watson SJ. Cellular localization and distribution of the cloned mu and kappa opioid receptors in rat gastrointestinal tract. *Neuroscience* 1997; **81**: 579-591
7 **Tavani A**, Gambino MC, Petrillo P. The opioid kappa-selective compound U-50,488H does not inhibit intestinal propulsion in rats. *J Pharm Pharmacol* 1984; **36**: 343-344
8 **Tavani A**, Petrillo P, La Regina A, Sbacchi M. Role of peripheral mu, delta and kappa opioid receptors in opioid-induced inhibition of gastrointestinal transit in rats. *J Pharmacol Exp Ther* 1990; **254**: 91-97
9 **La Regina A**, Petrillo P, Sbacchi M, Tavani A. Interaction of U-69,593 with mu-, alpha- and kappa-opioid binding sites and its analgesic and intestinal effects in rats. *Life Sci* 1988; **42**: 293-301
10 **Mitolo-Chieppa D**, Natale L, Marasciulo FL, De Salvatore G, Mitolo CI, Siro-Brigiani G, Renna G, De Salvia MA. Involvement of kappa-opioid receptors in peripheral response to nerve stimulation in kappa-opioid receptor knockout mice. *Auton Autacoid Pharmacol* 2002; **22**: 233-239
11 **Coupar IM**. The peristaltic reflex in the rat ileum: evidence for functional mu- and delta-opiate receptors. *J Pharm Pharmacol* 1995; **47**: 643-646
12 **Coupar IM**, De Luca A. Opiate and opiate antidiarrhoeal drug action on rat isolated intestine. *J Auton Pharmacol* 1994; **14**: 69-78
13 **Hancock DL**, Coupar IM. Evidence for functional delta-opiate receptors in the rat intestine. *J Pharm Pharmacol* 1994; **46**: 805-808
14 **Schiller PW**, Weltrowska G, Berezowska I, Nguyen TM, Wilkes BC, Lemieux C, Chung NN. The TIPP opioid peptide family: development of delta antagonists, delta agonists, and mixed mu agonist/delta antagonists. *Biopolymers* 1999; **51**: 411-425
15 **Schiller PW**. Development of receptor-specific opioid peptide analogues. *Prog Med Chem* 1991; **28**: 301-340
16 **Inturrisi CE**, Umans JG, Wolff D, Stern AS, Lewis RV, Stein S, Udenfriend S. Analgesic activity of the naturally occurring heptapeptide [Met]enkephalin-Arg6-Phe7. *Proc Natl Acad Sci USA* 1980; **77**: 5512-5514
17 **Majane EA**, Iadarola MJ, Yang HY. Distribution of Met5-enkephalin-Arg6, Phe7 in rat spinal cord. *Brain Res* 1983; **264**: 336-339
18 **Tang J**, Yang HY, Costa E. Inhibition of spontaneous and opiate-modified nociception by an endogenous neuropeptide with Phe-Met-Arg-Phe-NH2-like immunoreactivity. *Proc Natl Acad Sci USA* 1984; **81**: 5002-5005
19 **Galina ZH**, Kastin AJ. Existence of antiopiate systems as illustrated by MIF-1/Tyr-MIF-1. *Life Sci* 1986; **39**: 2153-2159
20 **Gupta S**, Pasha S, Gupta YK, Bhardwaj DK. Chimeric peptide of Met-enkephalin and FMRFa induces antinociception and attenuates development of tolerance to morphine antinociception. *Peptides* 1999; **20**: 471-478
21 **Gupta S**, Pasha S, Gupta YK, Bhardwaj DK. Effects of intracerebroventricularly administered chimeric peptide of metenkephalin and FMRFa--[D-Ala2]YFa-on antinociception and its modulation in mice. *Brain Res Bull* 2001; **55**: 51-57
22 **Vats ID**, Dolt KS, Kumar K, Karar J, Nath M, Mohan A, Pasha MA, Pasha S. YFa, a chimeric opioid peptide, induces kappa-specific antinociception with no tolerance development during 6 days of chronic treatment. *J Neurosci Res* 2008; **86**: 1599-1607
23 **Gupta K**, Vats ID, Gupta YK, Saleem K, Pasha S. Lack of tolerance and morphine-induced cross-tolerance to the analgesia of chimeric peptide of Met-enkephalin and FMRFa. *Peptides* 2008; **29**: 2266-2275
24 **Kumar K**, Kumar S, Kurupati RK, Seth MK, Mohan A, Hussain ME, Pasha S. Intracellular cAMP assay and Eu-GTP-γS binding studies of chimeric opioid peptide YFa. *Eur J Pharmacol* 2011; **650**: 28-33
25 **Vats ID**, Snehlata M, Pasha MA, Pasha S. Effect of chronic intra-peritoneally administered chimeric peptide of met-enkephalin and FMRFa-[D-Ala2]YFa-on antinociception and opioid receptor regulation. *Eur J Pain* 2010; **14**: 295.e1-295.e9
26 **Schiller PW**, Lipton A, Horrobin DF, Bodanszky M. Unsulfated C-terminal 7-peptide of cholecystokinin: a new ligand of the opiate receptor. *Biochem Biophys Res Commun* 1978; **85**: 1332-1338
27 **Valeri P**, Martinelli B, Morrone LA, Severini C. Reproducible withdrawal contractions of isolated guinea-pig ileum after brief morphine exposure: effects of clonidine and nifedipine. *J Pharm Pharmacol* 1990; **42**: 115-120
28 **Valeri P**, Morrone LA, Romanelli L. Manifestations of acute opiate withdrawal contracture in rabbit jejunum after mu-, kappa- and delta-receptor agonist exposure. *Br J Pharmacol* 1992; **106**: 39-44
29 **Ghosh MN.** Fundamentals of experimental pharmacology. 3rd ed. Kolkata: Hilton and Company, 2005: 110-120
30 **Bhargava HN**. Diversity of agents that modify opioid tolerance, physical dependence, abstinence syndrome, and self-administrative behavior. *Pharmacol Rev* 1994; **46**: 293-324
31 **Chavkin C**, Goldstein A. Demonstration of a specific dynorphin receptor in guinea pig ileum myenteric plexus. *Nature* 1981; **291**: 591-593
32 **Ferguson KM**, Higashijima T, Smigel MD, Gilman AG. The influence of bound GDP on the kinetics of guanine nucleotide binding to G proteins. *J Biol Chem* 1986; **261**: 7393-7399
33 **Wang D**, Surratt CK, Sadée W. Calmodulin regulation of basal and agonist-stimulated G protein coupling by the mu-

opioid receptor [OP(3)] in morphine-pretreated cell. *J Neurochem* 2000; **75**: 763-771
34 **Smith JA,** Leslie FM. Use of organ systems for opioid bioassay. Herz A, Akil H, Simon E, editors. Handbook of experimental pharmacology. Berlin: Springer-Verlag, 1993: 53-78
35 **Pound N**. Effects of morphine on electrically evoked contractions of the vas deferens in two congeneric rodent species differing in sperm competition intensity. *Proc Biol Sci* 1999; **266**: 1755-1758
36 **Vaught JL**, Rothman RB, Westfall TC. Mu and delta receptors: their role in analgesia in the differential effects of opioid peptides on analgesia. *Life Sci* 1982; **30**: 1443-1455
37 **Schoffelmeer AN**, Yao YH, Gioannini TL, Hiller JM, Ofri D, Roques BP, Simon EJ. Cross-linking of human [125I]beta-endorphin to opioid receptors in rat striatal membranes: biochemical evidence for the existence of a mu/delta opioid receptor complex. *J Pharmacol Exp Ther* 1990; **253**: 419-426
38 **Xu H**, Partilla JS, de Costa BR, Rice KC, Rothman RB. Differential binding of opioid peptides and other drugs to two subtypes of opioid delta ncx binding sites in mouse brain: further evidence for delta receptor heterogeneity. *Peptides* 1993; **14**: 893-907
39 **Traynor JR**, Elliott J. delta-Opioid receptor subtypes and cross-talk with mu-receptors. *Trends Pharmacol Sci* 1993; **14**: 84-86
40 **Maldonado R**, Severini C, Matthes HW, Kieffer BL, Melchiorri P, Negri L. Activity of mu- and delta-opioid agonists in vas deferens from mice deficient in MOR gene. *Br J Pharmacol* 2001; **132**: 1485-1492
41 **Raffa RB**, Stone DJ. Mu receptor and Gi2alpha antisense attenuate [D-Met2]-FMRFamide antinociception in mice. *Peptides* 1998; **19**: 1171-1175
42 **Takeuchi T**, Fujita A, Roumy M, Zajac JM, Hata F. Effect of 1DMe, a neuropeptide FF analog, on acetylcholine release from myenteric plexus of guinea pig ileum. *Jpn J Pharmacol* 2001; **86**: 417-422
43 **Decker B**, Vadokas B, Kutschenreuter U, Golenhofen K, Voigt K, McGregor GP, Mandrek K. Action of FMRFamide-like peptides on porcine gastrointestinal motility in vitro. *Peptides* 1997; **18**: 1531-1537
44 **Hutchinson M**, Kosterlitz HW, Leslie FM, Waterfield AA. Assessment in the guinea-pig ileum and mouse vas deferens of benzomorphans which have strong antinociceptive activity but do not substitute for morphine in the dependent monkey. *Br J Pharmacol* 1975; **55**: 541-546
45 **Karras PJ**, North RA. Acute and chronic effects of opiates on single neurons of the myenteric plexus. *J Pharmacol Exp Ther* 1981; **217**: 70-80
46 **Cohen ML**, Mendelsohn LG, Mitch CH, Zimmerman DM. Use of the mouse vas deferens to determine mu, delta, and kappa receptor affinities of opioid antagonists. *Receptor* 1994; **4**: 43-53
47 **Capasso A**. Comparison with naloxone of two dynorphin A analogues with K- and delta-opioid antagonist activity. *Med Chem* 2009; **5**: 1-6
48 **Metcalf MD**, Coop A. Kappa opioid antagonists: past successes and future prospects. *AAPS J* 2005; **7**: E704-E722
49 **Rozenfeld R**, Abul-Husn NS, Gomez I, Devi LA. An emerging role for the delta opioid receptor in the regulation of mu opioid receptor function. *Scient World J* 2007; **7**: 64-73

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BRIEF ARTICLE

Difference between CKD-EPI and MDRD equations in calculating glomerular filtration rate in patients with cirrhosis

Yu-Wei Chen, Han-Hsiang Chen, Tsang-En Wang, Ching-Wei Chang, Chen-Wang Chang, Chih-Jen Wu

Yu-Wei Chen, Han-Hsiang Chen, Chih-Jen Wu, Division of Nephrology, Department of Internal Medicine, Mackay Memorial Hospital, Taipei 10449, Taiwan
Yu-Wei Chen, Han-Hsiang Chen, Tsang-En Wang, Ching-Wei Chang, Chen-Wang Chang, Chih-Jen Wu, Mackay Medicine, Nursing and Management College, Taipei 10449, Taiwan
Han-Hsiang Chen, National Taipei College of Nursing, Taipei 10449, Taiwan
Tsang-En Wang, Ching-Wei Chang, Chen-Wang Chang, Division of Gastroenterology, Department of Internal Medicine, Mackay Memorial Hospital, Taipei 10449, Taiwan
Chih-Jen Wu, Graduate Institute of Medical Science, Taipei Medical University, Taipei 11031, Taiwan
Author contributions: Chen YW, Chen HH, Chang CW and Wu CJ designed the research; Chen YW, Chang CW and Chang CW performed the research; Chen YW and Chen HH contributed new reagents/analytic tools; Chen YW, Wang TE and Chang CW analyzed the data; and Chen YW wrote the paper.
Correspondence to: Dr. Chih-Jen Wu, Division of Nephrology, Department of Internal Medicine, Mackay Memorial Hospital, No. 92, sec 2, Zhongshan N Rd, Zhongshan Dist, Taipei 10449, Taiwan. yw.chen.mmh@gmail.com
Telephone: +886-2-25433535 Fax: +886-2-25433642
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Abstract

AIM: To evaluate the difference between the performance of the (CKD-EPI) and Modification of Diet in Renal Disease (MDRD) equations in cirrhotic patients.

METHODS: From Jan 2004 to Oct 2008, 4127 cirrhotic patients were reviewed. Patients with incomplete data with respect to renal function were excluded; thus, a total of 3791 patients were included in the study. The glomerular filtration rate (GFR) was estimated by the 4-variable MDRD (MDRD-4), 6-variable MDRD (MDRD-6), and CKD-EPI equations.

RESULTS: When serum creatinine was 0.7-6.8 mg/dL and 0.6-5.3 mg/dL in men and women, respectively, a significantly lower GFR was estimated by the MDRD-6 than by the CKD-EPI. Similar GFRs were calculated by both equations when creatinine was > 6.9 mg/dL and > 5.4 mg/dL in men and women, respectively. In predicting in-hospital mortality, estimated GFR obtained by the MDRD-6 showed better accuracy [81.72%; 95% confidence interval (CI), 0.94-0.95] than that obtained by the MDRD-4 (80.22%; 95%CI, 0.96-0.97), CKD-EPI (79.93%; 95%CI, 0.96-0.96), and creatinine (77.50%; 95%CI, 2.27-2.63).

CONCLUSION: GFR calculated by the 6-variable MDRD equation may be closer to the true GFR than that calculated by the CKD-EPI equation.

Key words: Chronic Kidney Disease Epidemiology Collaboration; Estimated glomerular filtration rate; Liver cirrhosis; Modification of Diet in Renal Disease; Renal function

Peer reviewer: Yogesh K Chawla, Dr., Professor, Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

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INTRODUCTION

Routine tests for serum creatinine (Scr) have been found to significantly improve the prognostic accuracy of

Child-Pugh score and to be an independent predictor of survival in patients with end-stage liver disease[1]. In the early 2000s, the Model for End-stage Liver Disease (MELD) score emerged as a simple and more objective score than Child-Pugh score, with Scr as one of the 3 variables included [the other 2 being international normalized ratio (INR) and serum bilirubin][2-4]. Unlike those of the Child-Pugh score, the 3 variables of the MELD score are selected on the basis of statistical analysis and not empirical analysis. Different from INR and serum bilirubin, which are the basic markers of liver function, Scr is essentially a marker of renal function; and highlights the prognostic significance of the interactions between liver and renal functions in patients with cirrhosis[5].

Kidney injury is an ominous and common event in cirrhotic patients[6]. Although Scr shows a strong prognostic value in patients with cirrhosis, it is considered an insensitive predictor in such patients because of reduced muscle mass, protein-deficient diet, severe hyperbilirubinemia, and diminished hepatic biosynthesis of serum creatinine, all of which lead to an overestimation of creatinine clearance as compared with inulin clearance[7]. Therefore, Scr level and creatinine-based equations also tend to overestimate glomerular filtration rate (GFR) in patients with cirrhosis.

Recently, a new creatinine-based equation known as the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation has been proposed as a more accurate formula than the Cockcroft and Modification of Diet in Renal Disease (MDRD) equations[8]. However, the CKD-EPI equation has not been tested in patients with cirrhosis. The aim of the present study was to evaluate the difference between the performance of the MDRD and CKD-EPI equations when evaluating renal function in a broader population of patients with cirrhosis than liver transplant registries.

MATERIALS AND METHODS

Patient information and data collection

A retrospective, cross-sectional, single-center study design was used, and the study protocol was approved by the local ethics committee. Patients diagnosed with cirrhosis were selected from those admitted to Mackay Memorial Hospital between January 2004 and October 2008.

Of a total of 228 345 admitted patients, the records of 4127 patients with cirrhosis were reviewed. Patients who survived and were followed up in the outpatient department were defined as survivors, and the most recent laboratory data available for them were collected. Patients whose records indicated death any time during the hospital stay were defined as non-survivors (cases of in-hospital mortality), and laboratory data for these patients were those collected during the admission in which death occurred. In the case of patients with multiple admissions, the records before those of the last admission were excluded. Demographic data, Child-Pugh scores, and information regarding underlying comorbidities were obtained from the most recent laboratory examinations. Patients with incomplete data with respect to Child-Pugh score and renal function or with cirrhosis due to congenital abnormality were excluded; thus, a total of 3791 patients were included in the study. None of the included patients had received liver transplants. The data on renal function in the common populace were based on the results of health examinations conducted among the residents of Taipei city, Taiwan, which were recently published as part of an epidemiologic study conducted at our institution[9].

Laboratory methods

We calibrated serum creatinine values using the modified Jaffe method (Beckman Coulter, Inc. UniCel® DxC 800 Synchron® Clinical System) which were further standardized using the isotope dilution mass spectrometry (IDMS) reference method at Mackay Memorial Hospital Laboratory

Equations

The GFR was calculated according to the listed formulae: MDRD-4 = $175 \times (\text{Scr})^{-1.154} \times (\text{Age})^{-0.203} \times (0.742$ if female) $\times (1.178$ if black)[10], MDRD-6 = $170 \times (\text{Scr})^{-0.999} \times (\text{Age})^{-0.176} \times (0.762$ if patient is female) $\times (1.180$ if black) $\times (\text{SUN})^{-0.170} \times (\text{Albumin})^{0.318}$[10], CKD-EPI = $141 \times \min(\text{Scr}/\kappa, 1)^{\alpha} \times \max(\text{Scr}/\kappa, 1)^{-1.209} \times 0.993^{\text{Age}} \times 1.018$ (if female) $\times 1.159$ (if black)[8],where MDRD-4 is the 4-variable MDRD, MDRD-6 is the 6-variable MDRD, age is given in years, albumin in g/dL, Scr is serum creatinine (mg/dL), SUN is serum urea nitrogen concentration (mg/dL), κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr/κ or 1, and max indicates the maximum of serum creatinine/κ or 1.

Statistical analysis

Continuous variables are summarized as mean ± standard deviation unless otherwise stated. We initially compared the demographic data and laboratory variables of survivors, non-survivors, and the common populace using the analysis of variance (ANOVA) test and chi-square test. Student's *t* test was used to assess differences in estimated GFR (eGFR) by CKD-EPI between cirrhotic patients and the common populace, and the difference in eGFR in cirrhotic patients calculated by MDRD6 or CKD-EPI, respectively. Logistic regression analyses were conducted to investigate the accuracy of predicting in-hospital mortality by the different creatinine-based equations. The results of these analyses were used to construct a receiver-operating characteristic (ROC) curve from which we sought the optimum cut-off point for predicting successful sites. The optimum cutoff point was defined as the point on the ROC curve closest to the point (0.1), where the false-positive rate was zero and the sensitivity was 100%. The area under the curve (AUC) and 95% confidence interval (CI) were

Table 1 Demographic and laboratory data of 3791 cirrhotic patients and 4292 common populace *n* (%)

Variables (*n*, %)	Survived cirrhotic patients (*n* = 2337)	Expired cirrhotic patients (*n* = 1454)	Common populace (*n* = 4292)	*P* value
Age (yr)	59.03 ± 14.03	63.61 ± 13.62	52.11 ± 12.13	< 0.001
Gender (male/female)	1620/717	990/464	2270/2022	< 0.001
Albumin (3.5-5 g/dL)	3.24 ± 0.68	2.48 ± 0.55	4.50 ± 0.29	< 0.001
BUN (8-12 mg/dL)	17.31 ± 14.77	60.81 ± 40.77	13.39 ± 3.78	< 0.001
Creatinine (0.4-1.2 mg/dL)	1.20 ± 1.06	2.93 ± 1.99	0.89 ± 0.20	< 0.001
eGFR (MDRD4)	79.27 ± 35.43	36.75 ± 33.55	81.72 ± 16.38	< 0.001
eGFR (MDRD6)	65.70 ± 30.28	26.76 ± 24.63	69.65 ± 13.16	< 0.001
eGFR (CKD-EPI)	78.50 ± 29.82	36.03 ± 30.23	88.31 ± 15.78	< 0.001
Total bilirubin (0.3-1.2 mg/dL)	2.24 ± 3.58	9.76 ± 10.68		< 0.001
INR	1.36 ± 0.43	2.70 ± 2.53		< 0.001
Hepatoma	647 (27.69)	717 (49.31)		< 0.001
Ascites	817 (34.96)	1018 (70.01)		< 0.001
Hepatic encephalopathy	431 (18.44)	649 (44.64)		< 0.001
Child-Pugh points	7.12 ± 1.97	10.37 ± 2.09		< 0.001

BUN: Blood urea nitrogen; eGFR: Estimated glomerular filtration rate; MDRD: Modification of Diet in Renal Disease; MDRD4: 4-variable MDRD; MDRD6: 6-variable MDRD: CKD-EPI: The Chronic Kidney Disease Epidemiology Collaboration; INR: International normalized ratio.

calculated. A *P* value of less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS software (version 17.0, SPSS Inc., Chicago, IL, United States).

RESULTS

Patient characteristics

Table 1 shows the demographic data, clinical characteristics, and laboratory data of patients with cirrhosis and the common populace. Older age, poorer renal function, and worse nutritional status were noted in the patients with cirrhosis than in the common populace. Thus, the average eGFR in patients with cirrhosis was significantly lower than that in the common populace, irrespective of the equation used for calculation (MDRD-4, MDRD-6, or CKD-EPI equation).

Difference between the performance of the MDRD-4, MDRD-6, and CKD-EPI equations in patients with cirrhosis and the common populace

Figure 1 shows the application of the 3 creatinine-based equations for calculating GFR in the common populace. The slope of the CKD-EPI equation was similar to that of the MDRD-4 equation when the Scr level was > 0.8 mg/dL and > 0.6 mg/dL in men and women, respectively, but less steep below the knots, which leads to less overestimation of GFR by the CKD-EPI equation at a lower Scr level[8]. Figure 2 shows the application of the CKD-EPI equation in calculating GFR in both the patients with cirrhosis and the common populace. At the same Scr level, the CKD-EPI equation tended to estimate a significantly lower value of GFR in patients with cirrhosis when the Scr level was 0.8-1.2 mg/dL and 0.5-1.1 mg/dL in men and women, respectively. Figure 3 shows the eGFR obtained by the 3 creatinine-based equations in patients with cirrhosis. The eGFRs obtained by the 3 equations were similar when the Scr level was > 6.9 mg/dL and > 5.4 mg/dL in men and women, respectively. Interestingly, significantly lower eGFR was obtained by the MDRD-6 equation than by the CKD-EPI equation when the Scr level was 0.7-6.8 mg/dL and 0.6-5.3 mg/dL in men and women, respectively. When the Scr level was < 0.5 mg/dL in men (1.8% of men with cirrhosis) and < 0.4 mg/dL in women (1.4% of women with cirrhosis), a lower eGFR was obtained by the CKD-EPI equation than by the MDRD-6 equation.

Prediction of in-hospital mortality by the different methods of renal function evaluation

The eGFR obtained by the MDRD-6 equation showed better accuracy (81.72%; 95% CI, 0.94-0.95) in predicting in-hospital mortality than that obtained by the MDRD-4 equation (80.22%; 95% CI, 0.96-0.97) and CKD-EPI equation (79.93%; 95% CI, 0.96-0.96). In general, eGFR showed a better prognostic value as a surrogate of renal function than Scr level (accuracy, 77.50%; 95% CI, 2.27-2.63). In the ROC curve (Figure 4), the cutoff point for eGFR obtained by the MDRD-6 equation was 41 (AUC, 0.85; 95% CI, 0.84-0.87). Interestingly, the cutoff point for Scr level was 1.3 mg/dL (AUC, 0.83; 95% CI, 0.81-0.84), which was lower than 1.5 mg/dL, a value suggested to indicate renal failure in patients with cirrhosis and the threshold value for the diagnosis of hepatorenal syndrome.

DISCUSSION

This retrospective, cross-sectional, single-center study involved a broader population of patients with cirrhosis than liver transplant registries to obtain eGFR using different creatinine-based equations. A significantly lower eGFR was obtained by the MDRD-6 equation than by the CKD-EPI equation when the Scr level was 0.7-6.8 mg/dL and 0.6-5.3 mg/dL in men and women, respectively. In view of the overall overestimation of GFR by the creatinine-based equations in patients with cirrhosis, eGFR obtained by the MDRD-6 equation may be closer to the

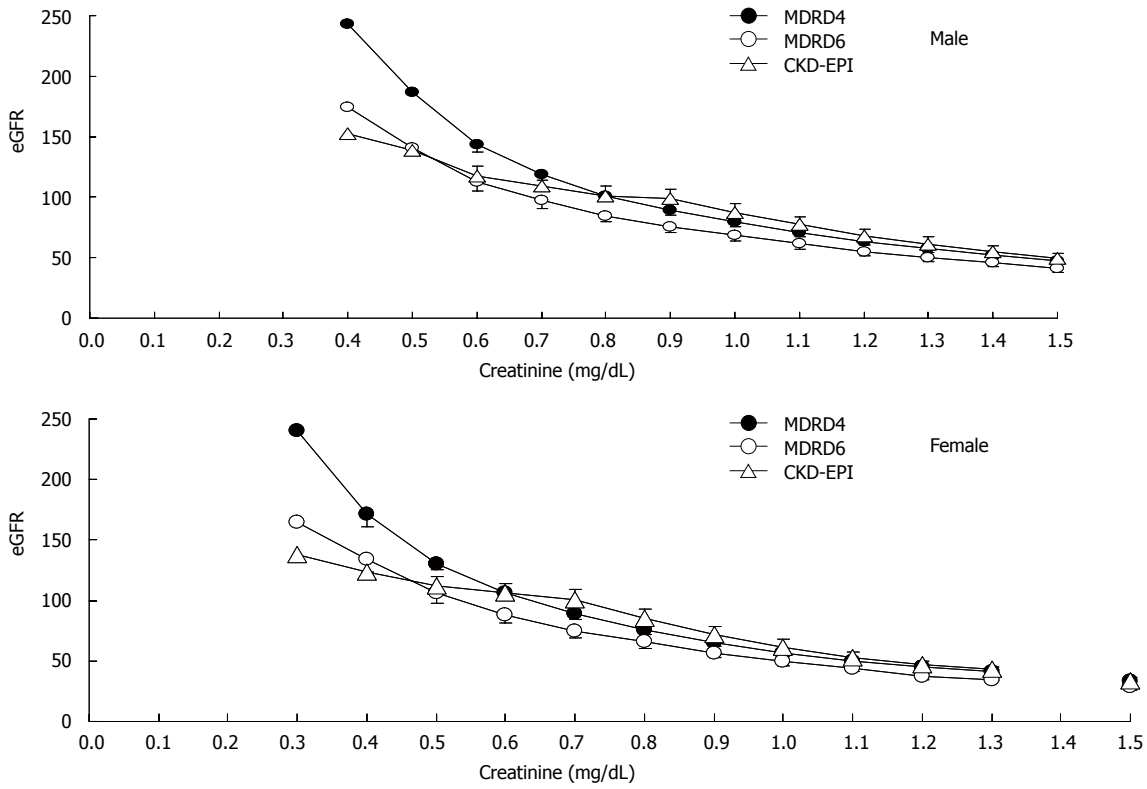


Figure 1 Estimated glomerular filtration rate obtained by the 4-variable Modification of Diet in Renal Disease, 6-variable Modification of Diet in Renal Disease, and the Chronic Kidney Disease Epidemiology Collaboration equations in the common populace. eGFR: Estimated glomerular filtration rate; MDRD: Modification of Diet in Renal Disease; MDRD4, 4-variable MDRD; MDRD6: 6-variable MDRD; CKD-EPI: The Chronic Kidney Disease Epidemiology Collaboration.

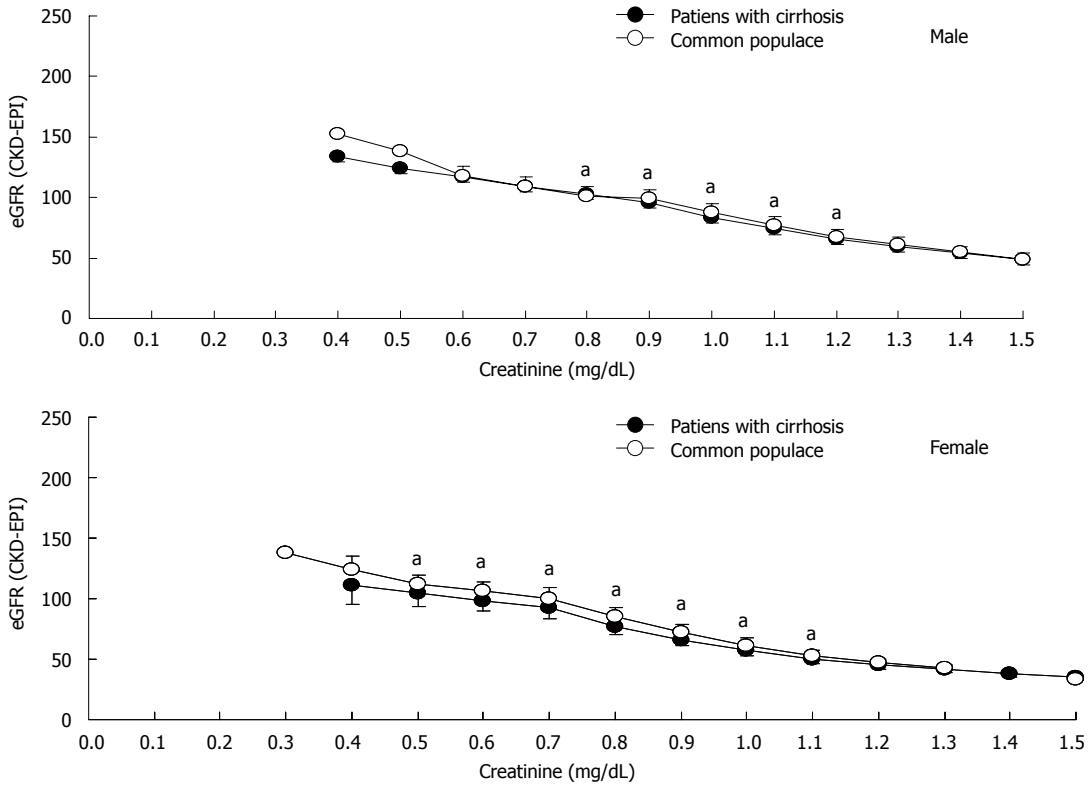


Figure 2 Estimated glomerular filtration rate obtained by the Chronic Kidney Disease Epidemiology Collaboration equation in patients with cirrhosis and the common populace. eGFR: Estimated glomerular filtration rate; CKD-EPI: The Chronic Kidney Disease Epidemiology Collaboration. ^{a}P <0.05, eGFR between the CKD-EPI *vs* 6-variable MDRD equations.

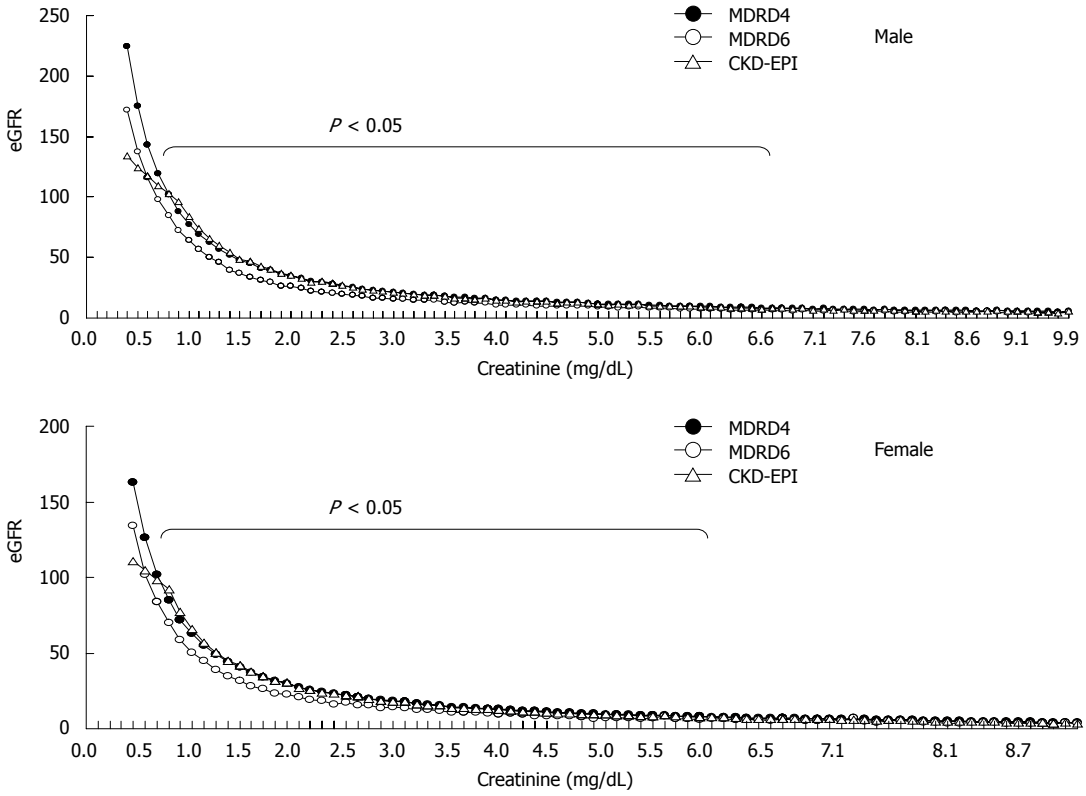
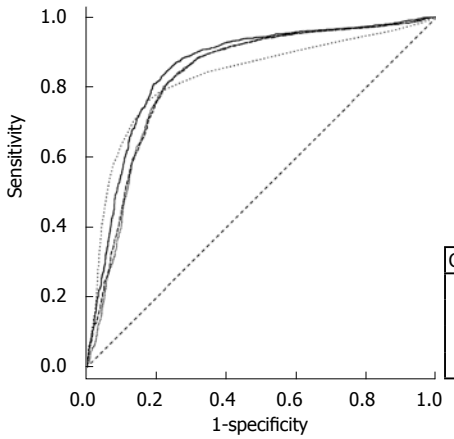


Figure 3 Estimated glomerular filtration rate obtained by the 4-variable Modification of Diet in Renal Disease, 6-variable Modification of Diet in Renal Disease, and the Chronic Kidney Disease Epidemiology Collaboration equations in patients with cirrhosis. eGFR: Estimated glomerular filtration rate; MDRD: Modification of Diet in Renal Disease; MDRD4: 4-variable MDRD; MDRD6: 6-variable MDRD; CKD-EPI: The Chronic Kidney Disease Epidemiology Collaboration.



Curve source	AUC	95% CI	Cut-off point	Sen	Spe
Cr	0.83	0.81-0.84	1.3	79%	79%
MDRD4	0.83	0.81-0.84	51-52	80%	78%
MDRD6	0.85	0.84-0.87	41	81%	81%
CKD-EPI	0.83	0.82-0.85	51-52	81%	77%

Figure 4 Receiver-operating characteristic curve of serum creatinine, 4-variable Modification of Diet in Renal Disease equation, 6-variable Modification of Diet in Renal Disease equation, and the Chronic Kidney Disease Epidemiology Collaboration equation for predicting in-hospital mortality. Cr: Creatinine; MDRD: Modification of Diet in Renal Disease; CKD-EPI: The Chronic Kidney Disease Epidemiology Collaboration; AUC: Area under curve; CI: Confidence interval; Sen: Sensitivity; Spe: Specificity.

true GFR than that obtained by the CKD-EPI equation. The use of eGFR obtained by the MDRD-6 equation as a surrogate of renal function offered better accuracy in predicting in-hospital mortality than that of eGFR obtained by the MDRD-4 equation, CKD-EPI equation, or Scr level.

The prognostic significance of renal function in patients with cirrhosis is reflected by the inclusion of Scr in the MELD score, which predicts short-term mortality (3 mo) and is used for the prioritization of transplant recipients in the United States[2-5,11]. However, it has recently been suggested that Scr weighs too heavily on the MELD score[12]: the assumption that mortality is constant at the Scr level of < 1 mg/dL is likely to be false. On the other hand, Scr level and creatinine-based equations tend to overestimate GFR, and creatinine clearance from the time of urine collection also leads to overestimation of GFR. As a result, a modified MELD score

with a lower weighting for Scr than that in the current MELD score has been proposed and has been shown to be slightly superior[12]. However, even after these adjustments, Scr is still a determinant of prognosis.

The creatinine-based Cockcroft and MDRD equations are widely used to estimate GFR in the general population, and MDRD is considered the gold standard in nephrology[5,13]. However, both the Cockcroft and MDRD equations tend to overestimate GFR: a series has shown that only 66% of estimates were within 30% of the measured GFR[14,15]. Unfortunately, most of the cited studies evaluated GFR in patients in liver transplant registries, who tend to have more advanced cirrhosis and decreased GFR, in part, due to the liver disease and malnourishment. The present study included a broader population that may have been better nourished or not as ill as that in previous studies.

The CKD-EPI equation, a newly developed equation for estimating GFR, has been proposed to be more accurate than the MDRD equation, especially when GFR is high. Moreover, it shows less bias, improved precision, and greater accuracy[8]. Our study results agreed with this fact since the slope of the CKD-EPI equation was less steep when the Scr level was < 0.8 mg/dL and < 0.6 mg/dL in men and women, respectively. When the CKD-EPI equation was applied at the same Scr level in patients with cirrhosis and the common populace, a lower GFR was calculated in the former than in the latter. This result was probably related to the older age of the patients with cirrhosis, with the same Scr level. When the CKD-EPI, MDRD-4, and MDRD-6 equations were applied in the case of patients with cirrhosis, the performance of the CKD-EPI and MDRD-4 equations was similar to that in the common populace. However, a significantly lower GFR was estimated by the MDRD-6 equation than by the CKD-EPI equation when the Scr level was 0.7-6.8 mg/dL and 0.6-5.3 mg/dL in men and women, respectively. This result was probably related to the higher blood urea nitrogen (BUN) and lower albumin level-the additional 2 variables used in the MDRD-6 equation-in patients with cirrhosis. Although the CKD-EPI equation also yielded a lower eGFR than the MDRD-6 equation when the Scr level was < 0.5 mg/dL and < 0.4 mg/dL in men and women, respectively, the value was only found in 1.8% men and 1.4% women in all the study subjects. In view of the overall overestimation of GFR by the creatinine-based equations in patients with cirrhosis, eGFR obtained by the MDRD-6 equation seemed to be closer to the true GFR than that obtained by the CKD-EPI equation.

Creatinine shows a significant prognostic value in patients with cirrhosis[2,5,11]. Theoretically, the creatinine-based equations show a similar prognostic value. However, the Cockcroft equation is less accurate than the MDRD equation since it incorporates body weight, which is markedly biased in patients with edema and/or ascites[16]. The MDRD-4 (simplified MDRD) equation is usually and most often used to calculate GFR, since it is considered as accurate as the original MDRD-6 equation[17]. However, its usefulness has not been proved in healthy individuals, and its accuracy may be low in specific clinical settings[15,18]. Therefore, the MDRD-6 equation is considered the best, possibly because it incorporates BUN and albumin level, the 2 variables which are abnormal in patients with cirrhosis[18]. Our data also showed that eGFR obtained by the MDRD-6 equation was more accurate than that obtained by the MDRD-4 equation, CKD-EPI equation, or even Scr level in predicting in-hospital mortality. It is most likely that the improved predication due to BUN and albumin, in particular serum albumin is an excellent predictor of mortality. Thus, the use of eGFR obtained by the MDRD-6 equation as a surrogate of renal function offers a better prognostic value than that of eGFR obtained by the other equations. However, the accuracy of the MDRD equation has only been estimated on a large scale, in patients with chronic kidney disease. This suggests that a specific formula should be derived for patients with cirrhosis.

The present study has several limitations. First, there was no comparison between the CKD-EPI equation and the gold standard for GFR estimation such as that using ^{125}I-iothalamate or inulin. Thus, the true performance of the CKD-EPI equation in patients with cirrhosis could not be evaluated. Second, due to the variation in assay of BUN and albumin across labs which is not standardized, these results may not be useful in other populations. Third, the study was retrospective and cross-sectional in nature, and therefore, a prospective, cohort study is needed to test and verify our conclusions.

In conclusion, in view of the overall overestimation of GFR in patients with cirrhosis by creatinine-based equations, GFR calculated by the MDRD-6 equation may be closer to the true GFR than that calculated by the CKD-EPI equation and, hence, more suitable as a surrogate of renal function. However, a formula specifically derived for calculating GFR in patients with cirrhosis is warranted.

COMMENTS

Background

The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation has been proposed to be more accurate than the Cockcroft and Modification of Diet in Renal Disease (MDRD) equations. However, the CKD-EPI equation has not been tested in patients with cirrhosis.

Research frontiers

This is a retrospective study. Glomerular filtration rate (GFR) calculated by the 6-variable MDRD equation is closer to the true GFR than that calculated by the CKD-EPI equation. The 6-variable MDRD equation is a better way of calculating GFR in cirrhotic patients.

Innovations and breakthroughs

To our knowledge, this is the first study to evaluate the difference between the performance of the CKD-EPI and MDRD equations in cirrhotic patients. Although the CKD-EPI equation been proposed to be more accurate than the MDRD equation in the general population, the 6-variable MDRD equation remains the best way to calculate GFR in cirrhotic patients.

Applications

GFR should be calculated by the 6-variable MDRD equation in cirrhotic patients.

Peer review

It is a good study. However the authors have adequately mentioned the limitation.

REFERENCES

1 **Abad-Lacruz A**, Cabré E, González-Huix F, Fernández-Bañares F, Esteve M, Planas R, Llovet JM, Quer JC, Gassull MA. Routine tests of renal function, alcoholism, and nutrition improve the prognostic accuracy of Child-Pugh score in nonbleeding advanced cirrhotics. *Am J Gastroenterol* 1993; **88**: 382-387

2 **Malinchoc M**, Kamath PS, Gordon FD, Peine CJ, Rank J, ter Borg PC. A model to predict poor survival in patients undergoing transjugular intrahepatic portosystemic shunts. *Hepatology* 2000; **31**: 864-871

3 **Kamath PS**, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, D'Amico G, Dickson ER, Kim WR. A model to predict survival in patients with end-stage liver disease. *Hepatology* 2001; **33**: 464-470

4 **Chen YW**, Wu CJ, Chang CW, Lee SY, Sun FJ, Chen HH. Renal function in patients with liver cirrhosis. *Nephron Clin Pract* 2011; **118**: c195-c203

5 **Francoz C**, Glotz D, Moreau R, Durand F. The evaluation of renal function and disease in patients with cirrhosis. *J Hepatol* 2010; **52**: 605-613

6 **Chen YW**, Wu CJ, Wang TE, Chang CW, Chang CW, Chen HH. The mortality survey of older patients with cirrhosis in Taiwan--a single-center experience. *J Am Geriatr Soc* 2010; **58**: 2230-2232

7 **Demirtas S**, Bozbas A, Akbay A, Yavuz Y, Karaca L. Diagnostic value of serum cystatin C for evaluation of hepatorenal syndrome. *Clin Chim Acta* 2001; **311**: 81-89

8 **Levey AS**, Stevens LA, Schmid CH, Zhang YL, Castro AF, Feldman HI, Kusek JW, Eggers P, Van Lente F, Greene T, Coresh J. A new equation to estimate glomerular filtration rate. *Ann Intern Med* 2009; **150**: 604-612

9 **Chen HH**, Chen YW, Wu CJ. Primary hyperparathyroidism in Taiwan: clinical features and prevalence in a single-center experience. *Endocrine* 2010; **37**: 373-378

10 **Levey AS**, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Ann Intern Med* 1999; **130**: 461-470

11 **Wiesner R**, Edwards E, Freeman R, Harper A, Kim R, Kamath P, Kremers W, Lake J, Howard T, Merion RM, Wolfe RA, Krom R. Model for end-stage liver disease (MELD) and allocation of donor livers. *Gastroenterology* 2003; **124**: 91-96

12 **Sharma P**, Schaubel DE, Sima CS, Merion RM, Lok AS. Reweighting the model for end-stage liver disease score components. *Gastroenterology* 2008; **135**: 1575-1581

13 **Stevens LA**, Coresh J, Greene T, Levey AS. Assessing kidney function--measured and estimated glomerular filtration rate. *N Engl J Med* 2006; **354**: 2473-2483

14 **Gonwa TA**, Jennings L, Mai ML, Stark PC, Levey AS, Klintmalm GB. Estimation of glomerular filtration rates before and after orthotopic liver transplantation: evaluation of current equations. *Liver Transpl* 2004; **10**: 301-309

15 **Cholongitas E,** Shusang V, Marelli L, Nair D, Thomas M, Patch D, Burns A, Sweny P, Burroughs AK. Review article: renal function assessment in cirrhosis - difficulties and alternative measurements. *Aliment Pharmacol Ther* 2007; **26**: 969-978

16 **Sherman DS**, Fish DN, Teitelbaum I. Assessing renal function in cirrhotic patients: problems and pitfalls. *Am J Kidney Dis* 2003; **41**: 269-278

17 **Levey AS**, Coresh J, Greene T, Stevens LA, Zhang YL, Hendriksen S, Kusek JW, Van Lente F. Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate. *Ann Intern Med* 2006; **145**: 247-254

18 **Myers GL**, Miller WG, Coresh J, Fleming J, Greenberg N, Greene T, Hostetter T, Levey AS, Panteghini M, Welch M, Eckfeldt JH. Recommendations for improving serum creatinine measurement: a report from the Laboratory Working Group of the National Kidney Disease Education Program. *Clin Chem* 2006; **52**: 5-18

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CASE REPORT

Closure of a persistent sphincterotomy-related duodenal perforation by placement of a covered self-expandable metallic biliary stent

Antonios Vezakis, Georgios Fragulidis, Constantinos Nastos, Anneza Yiallourou, Andreas Polydorou, Dionisios Voros

Antonios Vezakis, Georgios Fragulidis, Constantinos Nastos, Anneza Yiallourou, Andreas Polydorou, Dionisios Voros, 2nd Department of Surgery, Endoscopy Unit, Aretaieion Hospital, University of Athens, Athens 11528, Greece
Author contributions: Vezakis A and Polydorou A performed the endoscopies and contributed to study conception and design; Vezakis A, Fragulidis G, Nastos C and Yiallourou A contributed to research, analysis and interpretation of data; Vezakis A, Fragulidis G and Polydorou A wrote the paper; all authors revised the paper critically for important intellectual content; Voros D gave the final approval of the version to be published.
Correspondence to: Antonios Vezakis, MD, 2nd Department of Surgery, Endoscopy Unit, Aretaieion Hospital, University of Athens, 76 Vas Sofias Ave, Athens 11528, Greece. avezakis@hotmail.com
Telephone: +30-210-7286157 Fax: +30-210-9605145
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Abstract

Retroperitoneal duodenal perforation as a result of endoscopic biliary sphincterotomy is a rare complication, but it is associated with a relatively high mortality risk, if left untreated. Recently, several endoscopic techniques have been described to close a variety of perforations. In this case report, we describe the closure of a persistent sphincterotomy-related duodenal perforation by using a covered self-expandable metallic biliary (CEMB) stent. A 61-year-old Greek woman underwent an endoscopic retrograde cholangiopancreatography (ERCP) and sphincterotomy for suspected choledocholithiasis, and a retroperitoneal duodenal perforation (sphincterotomy-related) occurred. Despite initial conservative management, the patient underwent a laparotomy and drainage of the retroperitoneal space. After that, a high volume duodenal fistula developed. Six weeks after the initial ERCP, the patient underwent a repeat endoscopy and placement of a CEMB stent with an indwelling nasobiliary drain. The fistula healed completely and the stent was removed two weeks later. We suggest the transient use of CEMB stents for the closure of sphincterotomy-related duodenal perforations. They can be placed either during the initial ERCP or even later if there is radiographic or clinical evidence that the leakage persists.

Key words: Endoscopic sphincterotomy; Complications; Retroperitoneal perforation; Duodenal perforation; Metallic stent

Peer reviewer: Ji Kon Ryu, Professor, Department of Internal Medicine, Seoul National University College of Medicine, 28 Yeongeon-dong, Jongno-gu, Seoul 110-744, South Korea

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INTRODUCTION

Retroperitoneal duodenal perforation as a result of endoscopic biliary sphincterotomy (ES) is a well-recognized complication. Although it is reported to have an incidence of 0.3% to 1.3%, it is associated with a relatively high mortality rate of 7% to 14%[1-4]. The overriding question is whether or not immediate surgical exploration is required or if a trial of non-operative management is safe. Recently, several endoscopic techniques have been described to close a variety of perforations[5,6]. This is, to the best of our knowledge, the first report of a delayed closure of a sphincterotomy-related duodenal perforation by placement of a covered self-expandable metallic bili-

ary (CEMB) stent.

CASE REPORT

A 61-year-old lady with a history of previous laparoscopic cholecystectomy underwent endoscopic retrograde cholangiopancreatography (ERCP) for biliary colic, abnormal liver function tests and a grossly dilated common bile duct (20 mm). The suspected diagnosis was either choledocholithiasis or sphincter of Oddi dysfunction type I. ES was performed over a guidewire using an ERBE VIO 200 S diathermy (ERBE Elektromedizin, Germany) at Endocut mode. Due to an unusual direction of the papilla, the ES was carried out laterally towards the 9 o'clock position. After insertion and removal of the balloon catheter, a visible laceration was recognized just posteriorly to the ES. The presence of free air in the retroperitoneum was immediately apparent (Figure 1). The patient was treated initially with broad spectrum antibiotics and nasogastric drainage. The following day an abdominal computed tomography (CT) scan was performed (Figure 2). A large amount of retroperitoneal air was identified, but no leak of contrast was found. Over the following days the patient deteriorated, became pyrexial and unstable, and a laparotomy was carried out on the 15th post-ERCP day. The retroperitoneal space was explored with debridement of necrotic tissue and placement of drains.

The patient's condition was improved postoperatively, but a high volume duodenal fistula (500-1500 mL/24 h) developed, which was refractory to conservative treatment.

Four weeks later an endoscopy was performed. The procedure was technically difficult because of an edematous duodenum, but the laceration was found at the sphincterotomy site (Figures 3 and 4). A CEMB stent (Wallstent, Boston Scientific) and subsequently a nasobiliary drainage catheter were placed (Figures 5 and 6). The proximal 5 mm uncovered portion of the Wallstent had been cut prior to insertion. The fistula had healed completely a week later and the stent was removed at 2 wk. The nasobiliary drain was left in place for five days, although the output was minimal. A cholangiography and contrast study before removal of the nasobiliary drain showed no existence of leak.

The patient was discharged home 2 mo post-ERCP and remains well 8 mo later.

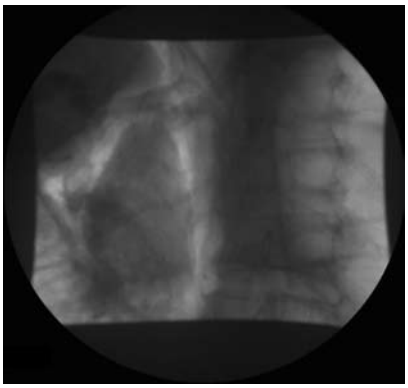

Figure 1 Free gas in the retroperitoneal space.

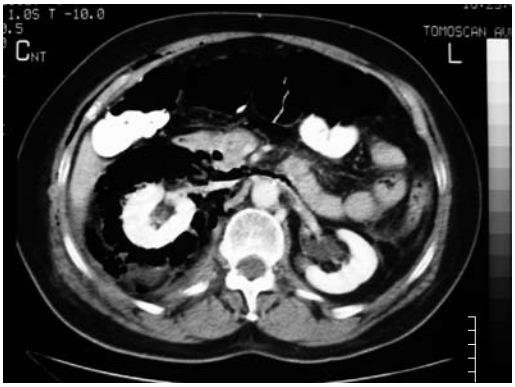

Figure 2 Computed tomography scan showing the presence of air in the retroperitoneal space and subcutaneous emphysema.

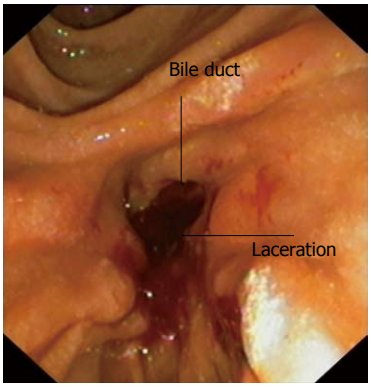

Figure 3 The laceration is evident just below the lower end of the bile duct.

DISCUSSION

Perforation during ES is usually retroperitoneal in location. It results from an extension of the incision beyond the intramural portion of the bile duct. It is widely believed that perforation is more likely to occur if the incision strays beyond the usual recommended sector (11 to 1 o'clock)[7]. Due to the low incidence of perforation, the risk factors are not well defined. The risk appears to be increased in patients with Billroth II anastomosis and when needle-knife sphincterotomy is performed[3]. The incision in these situations is not well controlled and therefore the chances of perforation are higher.

No consensus exists on management guidelines, because ES-related retroperitoneal perforations are rare and the clinical consequences vary enormously. In general, non-operative management of these perforations is possible despite the presence of extensive retroperitoneal air, provided the patient remains well clinically. If the patient develops abdominal pain, fever and appears toxic clinically, surgical exploration should be considered[8]. Delayed diagnosis can lead to severe morbidity[9]. Patients with biliary stenting appear to have a lower rate of operative intervention. This is likely related to the higher rate of guidewire-induced injuries in these patients, who are much less likely to require operative intervention[8].

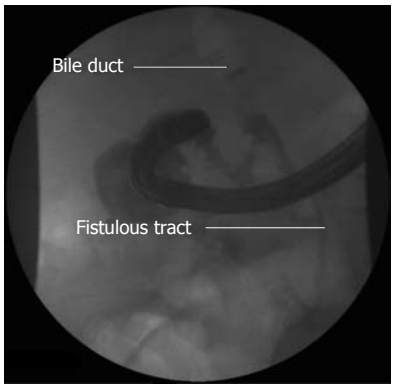

Figure 4 Injection of contrast from the endoscope enables visualization of the fistulous tract. The bile duct is also delineated with the presence of gas.

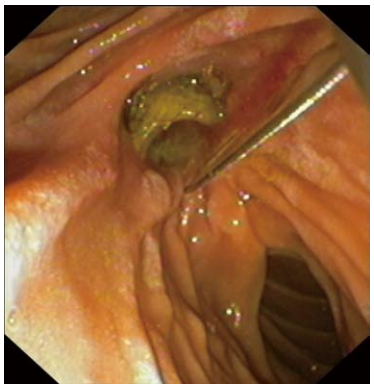

Figure 5 The covered self-expandable metallic biliary stent covers the laceration.

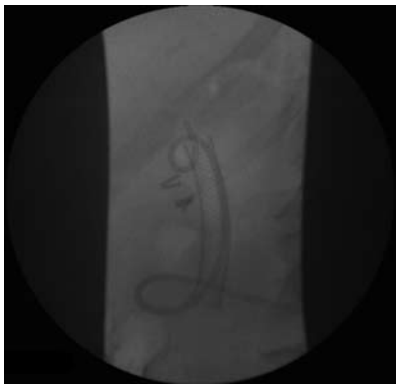

Figure 6 Covered self-expandable metallic biliary stent and nasobiliary catheter in place.

It is possible that biliary stenting has a protective effect by diverting bile into the duodenum instead of into the retroperitoneum. Endoscopic closure of ERCP-related duodenal perforations by using endoclipping devices[10], approximation sutures[5] or duodenal stents[6] have also been described.

The above described endoscopic techniques are applied after the recognition of the perforation during the initial ERCP. In our case, a repeat ERCP was performed 6 wk after the initial procedure, in order to repair the perforation and drain the bile duct. A repeat endoscopy after a duodenal perforation is technically demanding, carries the risk of extending the laceration, and excessive skill is required.

CEMB stents have the advantage of covering the laceration and permit free flow of bile into the duodenum instead of into the retroperitoneal space. Additionally, they protect against the leakage of pancreatic and gastric fluid. The additional use of a nasobiliary drainage catheter may reduce bile flow to the duodenum and allows checking of the healing process by performing contrast studies. Fully covered metallic self-expandable biliary stents should be preferred because they can easily be removed after a short period. In our case, a fully covered stent was not available and we modified a partially covered as mentioned previously.

In conclusion, we suggest the transient use of CEMB stents for the closure of sphincterotomy-related duodenal perforations. They can be placed either during the initial ERCP or even later, if there is radiographic or clinical evidence that the leakage persists.

REFERENCES

1 **Christensen M**, Matzen P, Schulze S, Rosenberg J. Complications of ERCP: a prospective study. *Gastrointest Endosc* 2004; **60**: 721-731
2 **Freeman ML**, Nelson DB, Sherman S, Haber GB, Herman ME, Dorsher PJ, Moore JP, Fennerty MB, Ryan ME, Shaw MJ, Lande JD, Pheley AM. Complications of endoscopic biliary sphincterotomy. *N Engl J Med* 1996; **335**: 909-918
3 **Loperfido S**, Angelini G, Benedetti G, Chilovi F, Costan F, De Berardinis F, De Bernardin M, Ederle A, Fina P, Fratton A. Major early complications from diagnostic and therapeutic ERCP: a prospective multicenter study. *Gastrointest Endosc* 1998; **48**: 1-10
4 **Fatima J**, Baron TH, Topazian MD, Houghton SG, Iqbal CW, Ott BJ, Farley DR, Farnell MB, Sarr MG. Pancreaticobiliary and duodenal perforations after periampullary endoscopic procedures: diagnosis and management. *Arch Surg* 2007; **142**: 448-454; discussion 454-455
5 **Lee TH**, Bang BW, Jeong JI, Kim HG, Jeong S, Park SM, Lee DH, Park SH, Kim SJ. Primary endoscopic approximation suture under cap-assisted endoscopy of an ERCP-induced duodenal perforation. *World J Gastroenterol* 2010; **16**: 2305-2310
6 **Small AJ**, Petersen BT, Baron TH. Closure of a duodenal stent-induced perforation by endoscopic stent removal and covered self-expandable metal stent placement (with video). *Gastrointest Endosc* 2007; **66**: 1063-1065
7 **Cotton PB**, Lehman G, Vennes J, Geenen JE, Russell RC, Meyers WC, Liguory C, Nickl N. Endoscopic sphincterotomy complications and their management: an attempt at consensus. *Gastrointest Endosc* 1991; **37**: 383-393
8 **Knudson K**, Raeburn CD, McIntyre RC, Shah RJ, Chen YK, Brown WR, Stiegmann G. Management of duodenal and pancreaticobiliary perforations associated with periampullary endoscopic procedures. *Am J Surg* 2008; **196**: 975-981; discussion 981-982
9 **Avgerinos DV**, Llaguna OH, Lo AY, Voli J, Leitman IM. Management of endoscopic retrograde cholangiopancreatography: related duodenal perforations. *Surg Endosc* 2009; **23**: 833-838
10 **Baron TH**, Gostout CJ, Herman L. Hemoclip repair of a sphincterotomy-induced duodenal perforation. *Gastrointest Endosc* 2000; **52**: 566-568

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Neoadjuvant plus adjuvant chemotherapy benefits overall survival of locally advanced gastric cancer

Xin-Zu Chen, Kun Yang, Jie Liu, Xiao-Long Chen, Jian-Kun Hu

Xin-Zu Chen, Kun Yang, Jie Liu, Jian-Kun Hu, Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Xiao-Long Chen, Faculty of Medicine, West China Medical School, Sichuan University, Chengdu 610041, Sichuan Province, China
Author contributions: Chen XZ designed this research and wrote the paper; Yang K, Liu J and Chen XL performed research; Hu JK is responsible for the academic inspection.
Correspondence to: Jian-Kun Hu, MD, PhD, Professor, Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, No. 37 Guoxue Xiang Street, Chengdu 610041, Sichuan Province, China. hujkwch@126.com
Telephone: +86-28-85422878 Fax: +86-28-85164035
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Peer reviewer: Robert C Moesinger, MD/FACS, Northern Utah Surgeons, Adjunct Assistant Professor, Department of Surgery, University of Utah, 4403 Harrison Blvd No. 1635, Ogden, UT 84403, United States

Chen XZ, Yang K, Liu J, Chen XL, Hu JK. Neoadjuvant plus adjuvant chemotherapy benefits overall survival of locally advanced gastric cancer. *World J Gastroenterol* 2011; 17(40): 4542-4544 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i40/4542.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i40.4542

Abstract

Neoadjuvant chemotherapy (NAC) has drawn more attention to the treatment of locally advanced gastric cancer (AGC) in the current multidisciplinary treatment model. EORTC trial 40954 has recently reported that NAC plus surgery without postoperative adjuvant chemotherapy could not benefit the locally AGC patients in their overall survival. We performed a meta-analysis of 10 studies including 1518 gastric cancer patients. Stratified subgroups were NAC plus surgery and NAC plus both surgery and adjuvant chemotherapy (AC), while control was surgery alone. The results showed that NAC plus surgery did not benefit the patients with locally AGC in their overall survival [odds ratio (OR) = 1.20, 95% CI 0.80-1.80, P = 0.37] and the number needed to treat (NNT) was 74. However, the NAC plus both surgery and AC had a slight overall survival benefit (OR = 1.33, 95% CI 1.03-1.71, P = 0.03) and NNT was 14, which is superior to the NAC plus surgery. Therefore, we recommend that combined NAC and AC should be used to improve the overall survival of the locally AGC patients.

TO THE EDITOR

We have read with great interest the excellent article by Li *et al*[1]. Gastric cancer is still one of the most common malignancies worldwide and about 80% patients with gastric cancer have advanced diseases[2]. Surgery is known as the only potentially curative treatment for this disease at resectable stages, while chemotherapy could play an important role in improving the prognosis of the patients[2,3]. Neoadjuvant chemotherapy (NAC) has drawn more attention to the treatment of locally advanced gastric cancer (AGC) in the current multidisciplinary treatment model. The EORTC trial 40954 observed the effect of the NAC without adjuvant chemotherapy (AC) following surgery for locally AGC (UICC stage Ⅲ and Ⅳ-cM0). The protocol did not benefit the survival of the patients, but the R0 resection rate was significantly increased[4]. However, the fluorouracil-containing AC for AGC has shown significant survival benefit compared with surgery alone[5-7]. Therefore, we consider that combined NAC and AC may benefit locally AGC patients in overall survival.

The meta-analysis performed by Li *et al*[1], including 14

Subgroup study	NAC Events	Total	Surgery alone Events	Total	Weigh	Odds ratio M-H, fixed, 95% CI	Year
NAC + S *vs* S							
Masuyama	18	24	58	74	16.3%	0.83 (0.28, 2.43)	1994
Wang	12	30	7	30	9.6%	2.19 (0.72, 6.70)	2000
Zhang	19	37	21	54	19.1%	1.66 (0.71, 3.86)	2004
Hartgrink	6	27	10	29	17.2%	0.54 (0.17, 1.78)	2004
Schyhmasher	40	72	37	72	37.8%	1.18 (0.61, 2.28)	2009
Subtotal (95% CI)		190		259	100.0%	1.20 (0.80, 1.80)	
Total events	95		133				
Heterogeneity: Chi^2 = 3.85, df = 4 (P = 0.43); I^2 = 0%							
Test for overall effect: Z = 0.90 (P = 0.37)							
NAC + S + AC *vs* S							
Nishioka	34	64	29	49	14.6%	0.78 (0.37, 1.66)	1982
Lygidakis	14	39	5	19	4.1%	1.57 (0.47, 5.27)	1999
Kobayashi	57	91	49	80	18.5%	1.06 (0.57, 1.97)	2000
Cunningham	101	250	83	253	46.7%	1.39 (0.96, 2.00)	2006
Boige	43	113	27	111	16.0%	1.91 (1.07, 3.40)	2007
Subtotal (95% CI)		557		512	100.0%	1.33 (1.03, 1.71)	
Total events	249		193				
Heterogeneity: Chi^2 = 4.08, df = 4 (P = 0.40); I^2 = 2%							
Test for overall effect: Z = 2.22 (P = 0.03)							

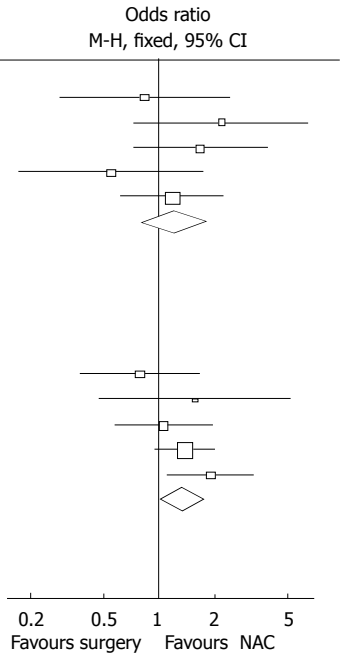

Figure 1 Subgroup comparison of neoadjuvant chemotherapy or neoadjuvant chemotherapy plus adjuvant chemotherapy *vs* surgery alone for locally advanced gastric cancer (based on the published meta-analysis and excluding the trials contaminated with adjuvant chemotherapy in control arm)[1]. M-H: Mantel-Haenszel test. NAC: Neoadjuvant chemotherapy; AC: Adjuvant chemotherapy.

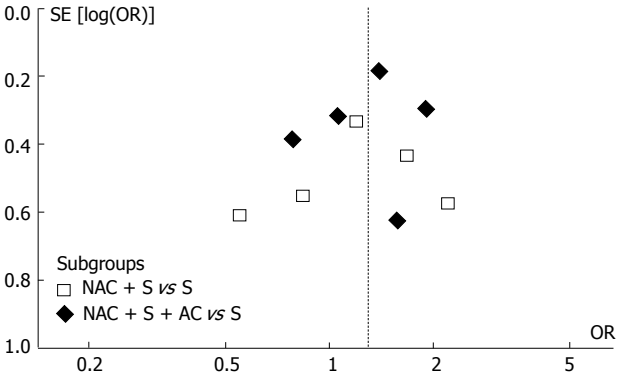

Figure 2 Funnel plot analysis of publication bias. OR: Odds ratio; NAC: Neoadjuvant chemotherapy; AC: Adjuvant chemotherapy.

trials, compared the patients treated with NAC and those without NAC, showed an R0 resection rate of 75.2% *vs* 66.9% [odds ratio (OR) = 1.51, P = 0.0006, fixed model]. Therefore, this made us well understand the substantial effectiveness of NAC for locally AGC.

Based on the original meta-analysis, we performed another subgroup analysis by classifying the intervention arms into NAC plus surgery or NAC plus both surgery and AC, while the trials contaminated with AC in the control arm were excluded[1]. In each subgroup, 5 prospective trials were included for repooled analysis (1518 patients). The meta-analysis showed that the NAC plus surgery did not bring overall survival benefit to the patients with locally AGC (OR = 1.20, 95% CI 0.80-1.80, P = 0.37) and the number needed to treat (NNT) was 74 (Figure 1). However, NAC plus both surgery and AC had a slight overall survival benefit (OR = 1.33, 95% CI 1.03-1.71, P = 0.03) and NNT was 14, which was superior to the NAC plus surgery (Figure 1). It implies that one out of 14 locally advanced patients treated by NAC plus both surgery and AC was benefited in survival, while the remainings might be at risk of recurrence or even death.

In addition, funnel plot observation did not indicate obvious publication bias in the two subgroups (Figure 2). The sensitivity analysis showed similar results by excluding the trials with Jadad score less than 3[1]. The original meta-analysis noted that the effect of NAC is more pronounced in Western countries and in doublet or triplet chemotherapy regimens[1]. In our subgroup analysis, the subgroups involved both Western and Asian studies as well as both single-agent and multi-agent regimens. These factors might not confound the present analysis.

Interestingly, EORTC trial 40 954 did not show any survival benefit by NAC for the locally advanced diseases (UICC stage Ⅲ and Ⅳ-cM0)[4], but the meta-analysis by Li *et al*[1] found that more advanced diseases (pT3-4) were benefited by NAC (OR = 1.91, P < 0.05) than pT1-2 diseases (P > 0.05). Based on the original data from Li *et al*[1], it is impossible to perform subgroup analysis of stage Ⅲ and Ⅳ-cM0 diseases, but it would be meaningful to figure out which sub-population might actually benefit from NAC plus both surgery and AC.

In summary, we think NAC is an effective therapy to increase the R0 resection rate for locally AGC, however, combined NAC and AC would improve the overall survival of the patients. Whether the increased R0 resection rate is associated with the improved overall survival and which sub-population might benefit from the combined

treatment requires further studies.

REFERENCES

1 **Li W**, Qin J, Sun YH, Liu TS. Neoadjuvant chemotherapy for advanced gastric cancer: a meta-analysis. *World J Gastroenterol* 2010; **16**: 5621-5628

2 **Chen XZ**, Jiang K, Hu JK, Zhang B, Gou HF, Yang K, Chen ZX, Chen JP. Cost-effectiveness analysis of chemotherapy for advanced gastric cancer in China. *World J Gastroenterol* 2008; **14**: 2715-2722

3 **Chen XZ**, Hu JK, Zhou ZG, Rui YY, Yang K, Wang L, Zhang B, Chen ZX, Chen JP. Meta-analysis of effectiveness and safety of D2 plus para-aortic lymphadenectomy for resectable gastric cancer. *J Am Coll Surg* 2010; **210**: 100-105

4 **Schuhmacher C**, Gretschel S, Lordick F, Reichardt P, Hohenberger W, Eisenberger CF, Haag C, Mauer ME, Hasan B, Welch J, Ott K, Hoelscher A, Schneider PM, Bechstein W, Wilke H, Lutz MP, Nordlinger B, Cutsem EV, Siewert JR, Schlag PM. Neoadjuvant chemotherapy compared with surgery alone for locally advanced cancer of the stomach and cardia: European Organisation for Research and Treatment of Cancer randomized trial 40954. *J Clin Oncol* 2010; **28**: 5210-5218

5 **Paoletti X**, Oba K, Burzykowski T, Michiels S, Ohashi Y, Pignon JP, Rougier P, Sakamoto J, Sargent D, Sasako M, Van Cutsem E, Buyse M. Benefit of adjuvant chemotherapy for resectable gastric cancer: a meta-analysis. *JAMA* 2010; **303**: 1729-1737

6 **Hu JK**, Li CM, Chen XZ, Chen ZX, Zhou ZG, Zhang B, Chen JP. The effectiveness of intravenous 5-fluorouracil-containing chemotherapy after curative resection for gastric carcinoma: A systematic review of published randomized controlled trials. *J Chemother* 2007; **19**: 359-375

7 **Hu JK**, Chen ZX, Zhou ZG, Zhang B, Tian J, Chen JP, Wang L, Wang CH, Chen HY, Li YP. Intravenous chemotherapy for resected gastric cancer: meta-analysis of randomized controlled trials. *World J Gastroenterol* 2002; **8**: 1023-1028

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Yeun-Jun Chung, MD, PhD, Professor, Director, Department of Microbiology, Integrated Research Center for Genome Polymorphism, The Catholic University Medical College, 505 Banpo-dong, Socho-gu, Seoul 137-701, Korea

Adrian G Cummins, Dr., Department of Gastroenterology and Hepatology, (DX 465384), 28 Woodville Road, Woodville South, 5011 South Australia, Australia

Gianpiero Gravante, MD, BsC, MBBS, Department of Hepatobiliary and Pancreatic Surgery, Leicester General Hospital, Flat 38, Room 8, Hospital Close, Leicester, LE5 4WU, United Kingdom

Alexander G Heriot, MA, MD, FRCS, FRACS, Associate Professor, Department of Surgical Oncology, Peter MacCallum Cancer Centre, 1 St Andrews Place, Melbourne, VIC 3002, Australia

Kevin Cheng-Wen Hsiao, MD, Assistant Professor, Colon and Rectal Surgery, Tri-Service General Hospital, No. 325, Sec 2, Cheng-Kung Rd, Nei-Hu district, 114 Taipei, Taiwan

Takashi Kojima, DVM, PhD, Department of Pathology, Sapporo Medical University School of Medicine, S1, W17, Chuo-ku, Sapporo 060-8556, Japan

Richard A Kozarek, MD, Department of Gastroenterology, Virginia Mason Medical Center, 1100 Ninth Avenue, PO Box 900, Seattle 98111-0900, United States

Michael Leitman, MD, FACS, Chief of General Surgery, Beth Israel Medical Center, 10 Union Square East, Suite 2M, New York, NY 10003, United States

Flavio Maina, PhD, Developmental Biology Institute of Marseille-Luminy, CNRS UMR 6216, Campus de Luminy-case 907, Marseille cedex 09, 13288, France

Satoshi Mamori, MD, PhD, Department of Gastroenterology and Hepatology, Shinko Hospital, 1-4-47 Wakihama-cho, Chuo-ku, Kobe, Hyogo 651-0072, Japan

Giulio Marchesini, Professor, Department of Internal Medicine and Gastroenterology, "Alma Mater Studiorum" University of Bologna, Policlinico S Orsola, Via Massarenti 9, Bologna 40138, Italy

Espen Melum, MD, Medical Department, Rikshospitalet University hospital, Sognsvannsvn 20, Oslo, 0027, Norway

Patrick O'Dwyer, MB, BCh, BAO, FRCS (1), MCh, FRCS (Glasg), University Department of Surgery,Western Infirmary, Glasgow, G11 6NT, United Kingdom

Alberto Piperno, Professor, Department of Clinical Medicine and Prevention, Clinical Medicine, San Gerardo Hospital, Via Pergolesi 33, 20052, Monza, Italy

John Plukker, MD, PhD, Professor of Surgical Oncology, Department of Surgery, University Medical Center groningen, Hanzeplein 1, Groningen, 9700 RB, The Netherlands

Adrian Saftoiu, MD, PhD, Professor, Research Center of Gastroenterology and Hepatology Craiova, University of Medicine and Pharmacy Craiova, 2 Petru Rares str, Craiova 200349, Romania

Henning Schulze-Bergkamen, MD, Henning Schulze-Bergkamen, First Medical Department, University of Mainz, Langenbeckstr, 1, 55101 Mainz, Germany

Bronislaw L Slomiany, PhD, Professor, Research Center, C-875, UMDNJ-NJ Dental School, 110 Bergen Street, PO Box 1709, Newark, NJ 07103-2400, United States

Hitoshi Tsuda, MD, PhD, Diagnostic Pathology Section,Clinical Laboratory Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

Vamsi R Velchuru, MRCS, FRCSEd, FRCS (Gen Surg), James Paget University Hospital, Great Yarmouth, 6 Pickwick Drive, Off Market Lane, Blundeston, NR32 5BX, United Kingdom

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Events Calendar 2011

January 14-15, 2011
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

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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Organization as author

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Both personal authors and an organization as author

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Volume with supplement

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Books

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Chapter in a book (list all authors)

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Conference proceedings

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Conference paper

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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

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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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