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Anorectal emergencies

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Abstract

Anorectal emergencies refer to anorectal disorders presenting with some alarming symptoms such as acute anal pain and bleeding which might require an immediate management. This article deals with the diagnosis and management of common anorectal

emergencies such as acutely thrombosed external hemorrhoid, thrombosed or strangulated internal hemorrhoid, bleeding hemorrhoid, bleeding anorectal varices, anal fissure, irreducible or strangulated rectal prolapse, anorectal abscess, perineal necrotizing fasciitis (Fournier gangrene), retained anorectal foreign bodies and obstructing rectal cancer. Sexually transmitted diseases as anorectal non-surgical emergencies and some anorectal emergencies in neonates are also discussed. The last part of this review dedicates to the management of early complications following common anorectal procedures that may present as an emergency including acute urinary retention, bleeding, fecal impaction and anorectal sepsis. Although many of anorectal disorders presenting in an emergency setting are not life-threatening and may be successfully treated in an outpatient clinic, an accurate diagnosis and proper management remains a challenging problem for clinicians. A detailed history taking and a careful physical examination, including digital rectal examination and anoscopy, is essential for correct diagnosis and plan of treatment. In some cases, some imaging examinations, such as endoanal ultrasonography and computerized tomography scan of whole abdomen, are required. If in doubt, the attending physicians should not hesitate to consult an expert *e.g.*, colorectal surgeon about the diagnosis, proper management and appropriate follow-up.

Key words: Anorectal; Emergencies; Hemorrhoid; Fissure; Abscess; Rectal prolapse; Sepsis; Complication; Sexually transmitted disease; Imperforate anus; Rectal cancer

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Core tip: Anorectal emergencies refer to anorectal disorders presenting with acute symptoms and signs which might require an immediate management. Anorectal emergencies usually include acutely thrombosed external hemorrhoid, complicated internal hemorrhoid, anal fissure, irreducible rectal prolapse,

anorectal sepsis, sexually transmitted proctitis, obstructing rectal cancer and early complications after anorectal procedures. A detailed history taking, careful physical examination including digital rectal examination and anoscopy, and some radiological imaging are essential for correct diagnosis and plan of treatment. Clinicians should be familiar with these conditions especially anorectal sepsis which could be potentially lethal if delay in diagnosis and management.

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INTRODUCTION

Anorectal emergencies refer to anorectal disorders presenting with some alarming symptoms such as anorectal pain and bleeding which might require an immediate management. Anorectal emergencies include acutely thrombosed external hemorrhoid, complicated internal hemorrhoid, anal fissure, anorectal sepsis, irreducible rectal prolapse, sexually transmitted proctitis and obstructing rectal cancer. Although most of these conditions are not life-threatening and may be successfully treated in an outpatient setting, an accurate diagnosis remains a challenging problem for physicians and surgeons^[1]. It should be noted that patients with acute anorectal problem should be handled with a careful clinical assessment since many of them are suffering from pain, discomfort and embarrassment. If necessary, rectal examination may be performed under anesthesia. In some cases, some imaging examinations, such as endoanal ultrasonography and computerized tomography (CT) scan, are required to confirm diagnosis and plan for treatment. A delay to diagnosis or appropriate treatment of these anorectal disorders was associated with poor outcomes^[2]. A referral or consultation should be made to surgeon if an operation is, or may be, needed. This paper summarizes the diagnosis and treatment of common anorectal emergencies excluding anorectal trauma. The last part of this review dedicates to the management of early complications following common anorectal procedures that may present as an emergency.

ACUTELY THROMBOSED EXTERNAL HEMORRHOID

Diagnosis

Classic symptoms of this condition are acute anal pain with a newly enlarged or tender bluish lump at the anal verge. Some patients may give a history of recent constipation or prolonged straining. Acutely

thrombosed external hemorrhoid usually causes severe pain in the first couple of days and the pain will gradually subside thereafter. High pressure within the thrombus may cause the erosion of overlying skin and thus resulting in bleeding. Acutely thrombosed external hemorrhoid must be differentiated from complicated internal hemorrhoids and, sometimes, from anal pigmented melanoma. A practical point is that the former is covered by anoderm and the clot formation is lying beneath the skin (Figure 1A and B). In contrary, internal hemorrhoid is covered by anal mucosa and anal pigmented melanoma presents with a longer history of dark pigmented skin lesion (Figure 1C and D).

Management

Acutely thrombosed external hemorrhoid can be treated conservatively or surgically depending on patient's symptoms (mainly the intensity of present pain). Basically, excision of thrombosed external hemorrhoid or surgical removal of clot is reserved in patients experiencing severe pain - usually within 48-72 h of onset. Otherwise, conservative management would be offered including anti-inflammatory analgesics, warm sitz bath, reducing activity and avoiding constipation. Education and reassurance about this condition and its benign nature would be beneficial to the patient.

THROMBOSED OR STRANGULATED INTERNAL HEMORRHOID

Diagnosis

Internal hemorrhoid may become strangulated and thrombosed when prolapsed part is left protruded until vascular compromise or venous stasis occurs. Patients with acute thrombosis and strangulation of internal hemorrhoids usually present with acute irreducible and painful hemorrhoid. Foul-smelling discharge may be seen in those with mucosal necrosis.

Management

This condition is difficult to manage especially in case of extensive thrombosis and strangulation. Manual reduction of the hemorrhoid masses might help in reducing pain and tissue congestion. Urgent hemorrhoidectomy is usually required^[3] (Figure 2). Some technical notes of hemorrhoidectomy in this situation are listed in Table 1.

BLEEDING HEMORRHOID

Diagnosis

Bleeding from hemorrhoid is characterized by a painless passage of bright-red blood during bowel movements, with or without prolapsed hemorrhoid. The blood may be spotted on toilet paper after cleansing or drip into toilet bowl. Bleeding tends

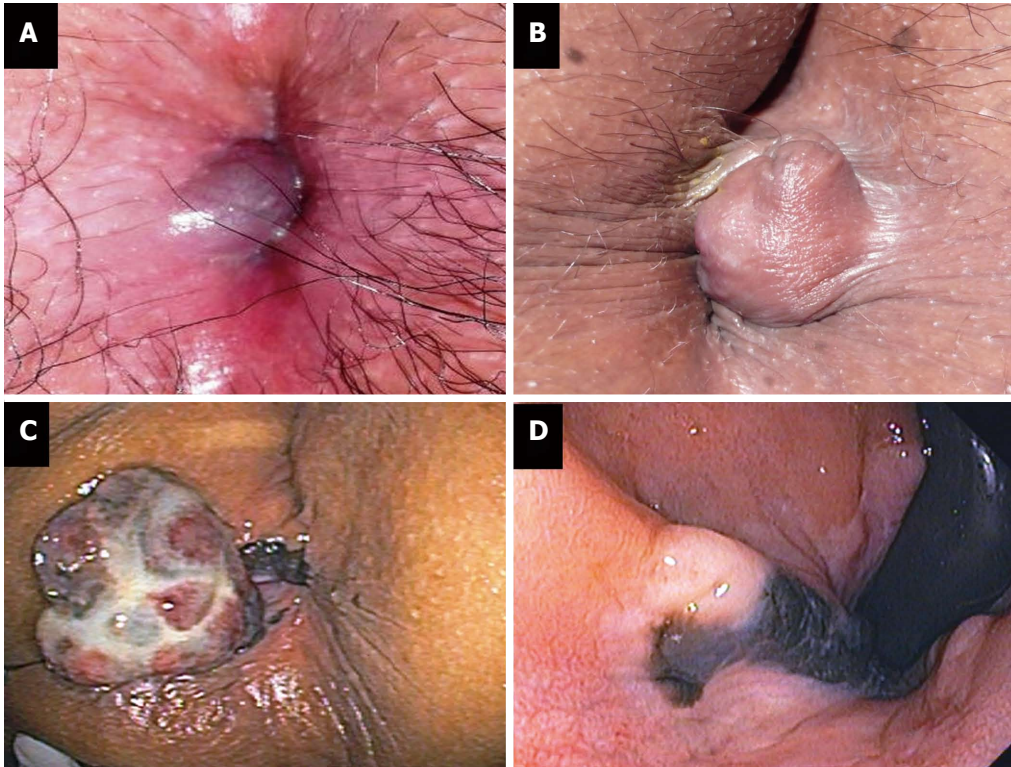


Figure 1 Acutely thrombosed external hemorrhoid (A, B) and anal melanoma (C, D).

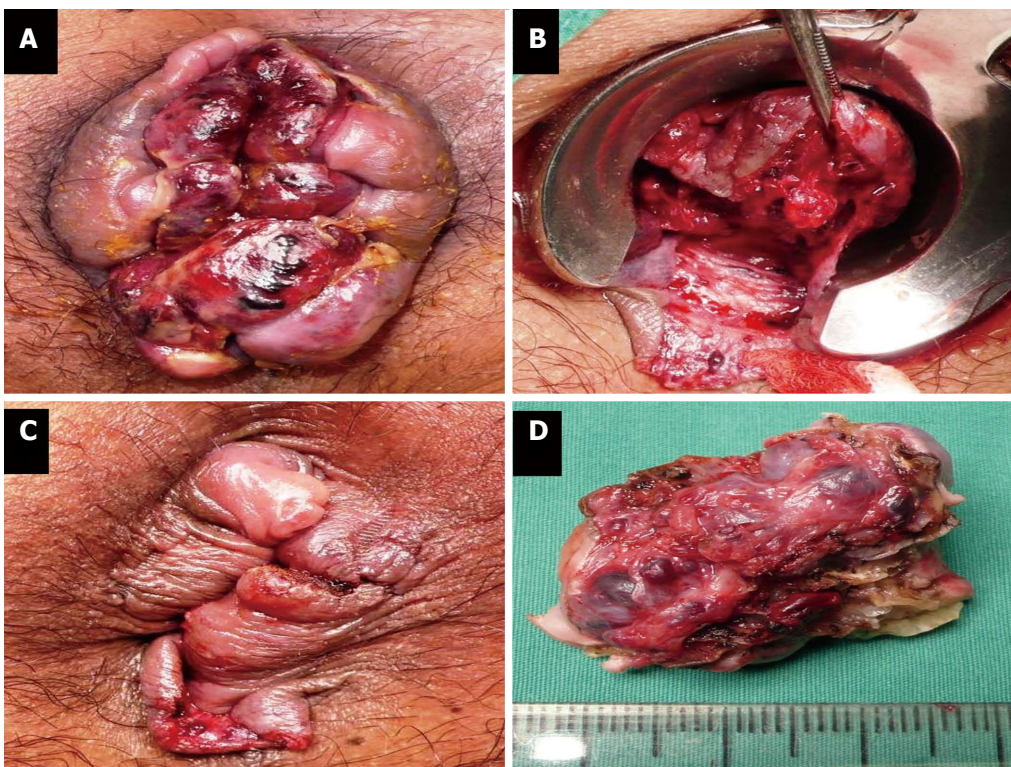


Figure 2 Urgent hemorrhoidectomy for thrombosed internal hemorrhoids (A-D).

to be mild except individuals having antiplatelet or anticoagulant therapy. Differential diagnoses include anal fissure (which is associated with painful defecation) and bleeding rectal neoplasm. The

diagnosis can be confirmed by a typical history, digital rectal examination and anoscopy - which usually reveal some stigmata of recent bleeding on hemorrhoid tissue.

Table 1 Technical notes for urgent hemorrhoidectomy

| |
|--|
| Preoperative intravenous antibiotics |
| Surgery under general anesthesia, regional anesthesia, or intravenous sedation plus perianal infiltration of local anesthetic agent(s) |
| Prone jackknife position |
| Manual reduction of prolapsing hemorrhoids |
| Compression of hemorrhoids to reduce edema |
| During an operation, use of large-diameter anoscope <i>e.g.</i> , Fansler anoscope |
| Anoderm or mucosa-sparing hemorrhoidectomy (preferably semi-closed technique) |
| Allowance of at least 1-cm mucosal bridge between surgical wounds and at least 50% of good circumferential mucosa |
| Use of long-lasting absorbable sutures <i>e.g.</i> , polyglactin 910 for mucosal approximation |
| If applicable, instead of hemorrhoidectomy, plication of hemorrhoid may be applied to small lesions |
| Oral postoperative antibiotics against anaerobes for 1 wk |

Management

Choices of treatment depend on the degree of bleeding, grade of hemorrhoid, patient's comorbidity and patient's preference^[4]. For low-graded hemorrhoid, management includes dietary and lifestyle modification, avoidance of constipation or diarrhea, topical medication, oral venotonic drug, and some office-based procedures *e.g.*, rubber band ligation and injection sclerotherapy. For high-graded hemorrhoid, surgical management may be offered including hemorrhoidectomy, dopper-guided hemorrhoidal artery ligation and stapled hemorrhoidopexy. The management of bleeding hemorrhoids in complicated situations (such as pregnancy, immunocompromised host and patients having antiplatelet or anticoagulant therapy) has been recently reviewed in this journal^[5].

BLEEDING ANORECTAL VARICES**Diagnosis**

Anorectal bleeding in patients with a history of long-standing or uncontrolled portal hypertension would give a clinician clues about this condition. However, hemorrhoid is more prevalent in such patients^[6]. It is important to differentiate bleeding hemorrhoids from bleeding anorectal varices because the choices of treatment are different. Practically, diagnosis and differentiation between the two conditions is best achieved with anoscopy or flexible sigmoidoscopy. Since hemorrhoid is an abnormal anal cushion with dilatation of hemorrhoid venous plexus, it is located within the anal canal^[4]. On the other hand, anorectal varices - dilated submucosal veins of portosystemic collateral circulation^[7] - can be visualized as enlarged and tortuous submucosal veins extended from the anal canal up to the middle rectum.

Management

The management of bleeding anorectal varices can be very challenging. In mild cases, intravenous

fluid replacement, blood transfusion, correction of coagulopathy and optimal medication for portal hypertension is usually effective. In active variceal bleeding, per anal suture ligation along the course of varices, endoscopic ligation of the varices and injection sclerotherapy are helpful^[3]. In severe or recurrent cases, a decrease in portal pressure is an ultimate goal of treatment which can be achieved by means of surgical portosystemic shunt or preferably transjugular intrahepatic portosystemic shunt (TIPS)^[8].

ANAL FISSURE**Diagnosis**

Painful defecation with a passage of red blood is a typical symptom of this condition. Pain is usually excruciating and may last from minutes to several hours. Although patients are relatively pain-free between bowel movement, experiencing severely painful defecation may preclude patients to have another bowel movement resulting in even harder stool. A vicious cycle of pain, anal spasm and passage of hard stool would exacerbate further traumatic and ischemic injury to anoderm and prevent the fissure from healing.

For those with a short history of painful defecation, a small shallow linear laceration of the anoderm in the midline (acute anal fissure) is normally evident without the need of digital rectal examination. Meanwhile, a chronic linear laceration of anoderm exposure to the underlying internal anal sphincter, with or without hypertrophic anal papilla and enlarged perianal skin tag, is a paramount finding of chronic anal fissure.

Management

Acute anal fissure usually heal within a few week by means of conservative treatment - which includes adequate pain control, stool softeners, laxative and warm sitz bath. Patient education is also essential to prevent or minimize disease recurrence. Medication that reduce anal sphincter tone may be prescribed in patients with acute or chronic anal fissure such as topical nitrate and topical calcium channel blocker^[9]. Although lateral internal anal sphincterotomy remains a standard treatment for chronic anal fissure^[10], botulinum toxin injection is an effective alternative to surgery especially in those with coexisting anal incontinence or anal sphincter hypotonia.

IRREDUCIBLE OR STRANGULATED RECTAL PROLAPSE**Diagnosis**

First, clinicians should differentiate prolapsed rectum from circumferentially prolapsed internal hemorrhoid. Classic signs of rectal prolapse are protruding full-thickness rectal wall with concentric rings of mucosa (Figure 3A), while hemorrhoid contains only mucosa

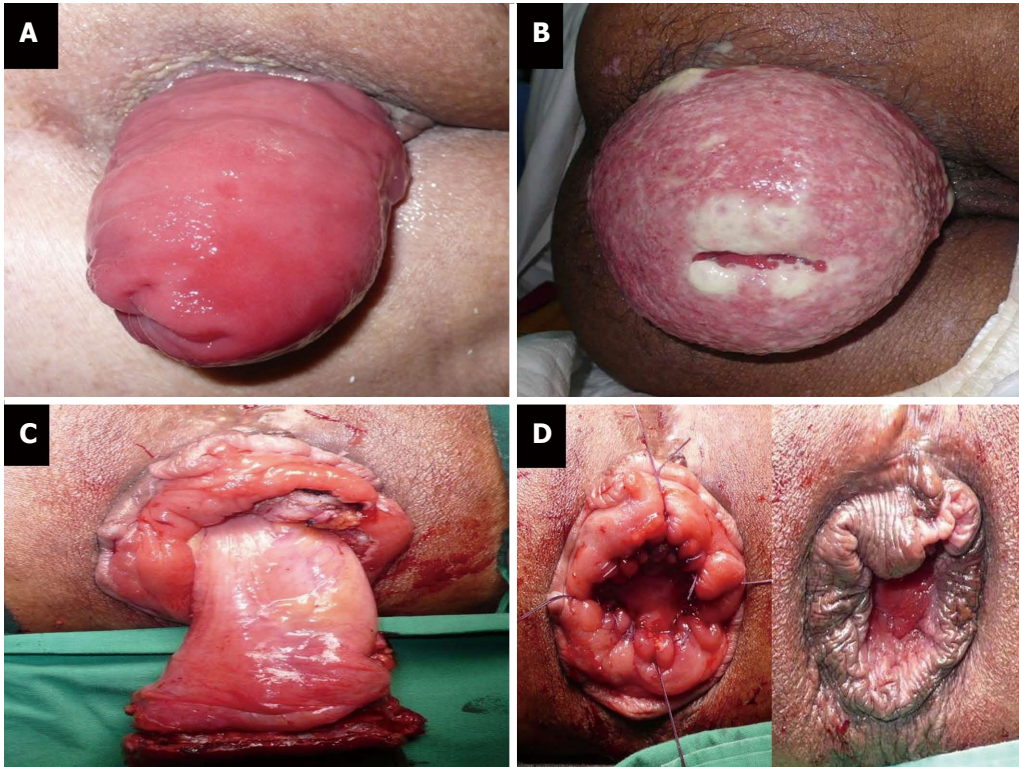


Figure 3 Rectal prolapse (A), strangulated rectal prolapse (B), and perineal rectosigmoidectomy or Altemeier's procedure (C, D).

and there are radial sulci between hemorrhoid bundles. Irreducible rectal prolapse may occur but acute strangulation of rectal prolapse is quite rare (Figure 3B). Nevertheless, both conditions require prompt intervention.

Management

Although a range of techniques and approaches have been described to treat reducible rectal prolapse^[11], perineal rectosigmoidectomy (Altemeier's procedure) is the treatment of choice in strangulated rectal prolapse (Figure 3C and D). For irreducible non-strangulated rectal prolapse, gentle reduction under intravenous sedation and analgesia is helpful and definitive surgery can be deferred^[12].

ANORECTAL ABSCESS

Diagnosis

An abscess forming in the anorectal region usually originates from an infected anal gland which is located in the anal mucosa and its opening is at the level of dentate line. Once the anal gland is infected, an abscess may form within an intersphincteric area or it could spread to an adjacent area such as perianal region, deep postanal space, ischioanal fossa or, rarely, a supralelevator space. Acute anorectal abscess may be an initial manifest of anal fistula.

Most anorectal abscesses can be readily diagnosed by a careful history and physical examination. The leading symptom of anorectal abscess is acute and

throbbing anal pain, which may be aggravated by coughing and sitting. Tender lump or swelling of affected area, with or without fever, is commonly seen. Fluctuation of the abscess is usually minimal or not evident in the anorectal abscess because of loose fatty connective tissue in this area. In case of an intersphincteric abscess or a suprasphincteric abscess, perianal inspection could appear normal but digital rectal examination often reveals a painful bulging area of the abscess. Three-dimensional endoanal ultrasonography (Figure 4) or CT scan and MRI of the pelvis may give some additional information on the location and extension of the abscess^[13,14].

Management

The goal of treatment is to provide an adequate and dependent drainage of the abscess. For perianal abscess and ischioanal (ischioanal) abscess, an elliptical or cruciate incision will be performed over the abscess to provide an adequate drainage and to prevent a premature closure of the incision. If possible, the drainage should be performed close to the anal verge because it will shorten the length of potential subsequent fistula tract^[15]. An intersphincteric approach is used for surgical drainage of intersphincteric abscesses - with or without the division of internal anal sphincter. With the guidance of diagnostic imaging modalities, drainage of supralelevator abscess can be performed by transrectal approach, intersphincteric approach, and trans-ischioanal approach depending on the origin of

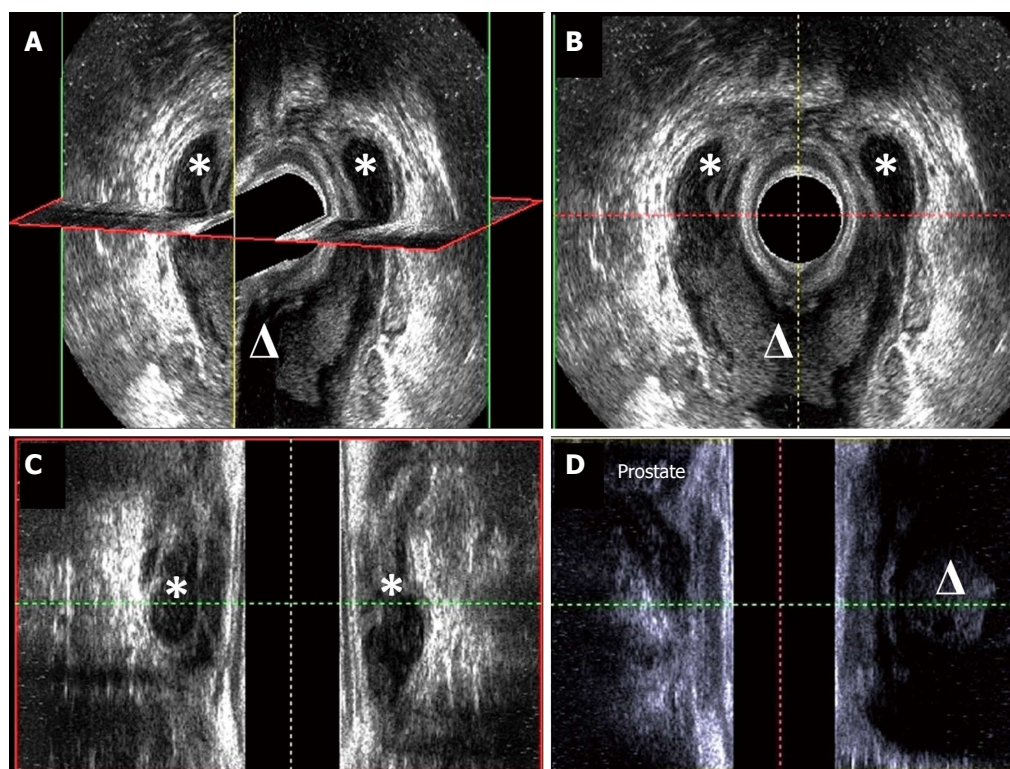


Figure 4 Three-dimensional endoanal ultrasonography of horseshoe abscess (A), cross-sectional view (B), coronal view (C) and sagittal view (D). The asterisk means abscess in ischiorectal space and the triangle means abscess in deep postanal space.

infection. Transrectal drainage is used for supralelevator abscess with intact pelvic floor muscle. Intersphincteric drainage and trans-ischioanal drainage are used for supralelevator abscess originated from intersphincteric space and ischioanal fossa, respectively.

The addition of intravenous antibiotics to surgical drainage is recommended in patients with extensive overlying cellulitis, immunocompromised hosts, those with concomitant systemic illness and those with prosthetic heart valves^[16]. The management of anal fistula-associated anorectal abscess remains controversial. An experienced surgeon may provide a definite treatment of anal fistula in this situation. However, it is widely accepted that the abscess can be drained first and then a schedule is made for fistula management in another setting^[17]. Treatment following drainage of abscess usually includes warm sitz bath, adequate pain control and the prevention of constipation. The patients should be advised about an approximately 30% chance of anal fistula formation following incision and drainage^[15].

PERINEAL NECROTIZING FASCIITIS (FOURNIER GANGRENE)

Diagnosis

Perineal necrotizing fasciitis is a severe and life-threatening form of skin and soft tissue infection in

the anal and perineal region. It is usually polymicrobial infection that develops secondary to untreated anorectal abscess, genitourinary infection or cutaneous infection^[18]. It is more likely to occur in diabetic individuals and immunocompromised hosts. Patient with perineal necrotizing fasciitis is characterized by severe perineal pain and high-graded fever. Septic shock and acute urinary retention may develop. On a physical examination, markedly swelling of buttock and perineum, with or without purple bullae and necrosis of overlying skin, is a hallmark feature. The most important tool for early diagnose this condition is to have a high index of suspicion. CT scan of lower abdomen may be useful in the diagnosis and delineation of the extension of perineal necrotizing fasciitis^[19].

Management

Intravenous fluid resuscitation and broad spectrum intravenous antibiotics must be given. Prompt and adequate surgical debridement of infected tissue is the mainstay of treatment (Figure 5). A delay in treatment would have a negative impact on patient's survival^[20]. Diverting colostomy may be performed to reduce fecal contamination and to facilitate perineal wound healing. Several reconstructive procedures, such as vacuum-assisted closure, skin graft and myocutaneous flap, can be used to correct the tissue defect.

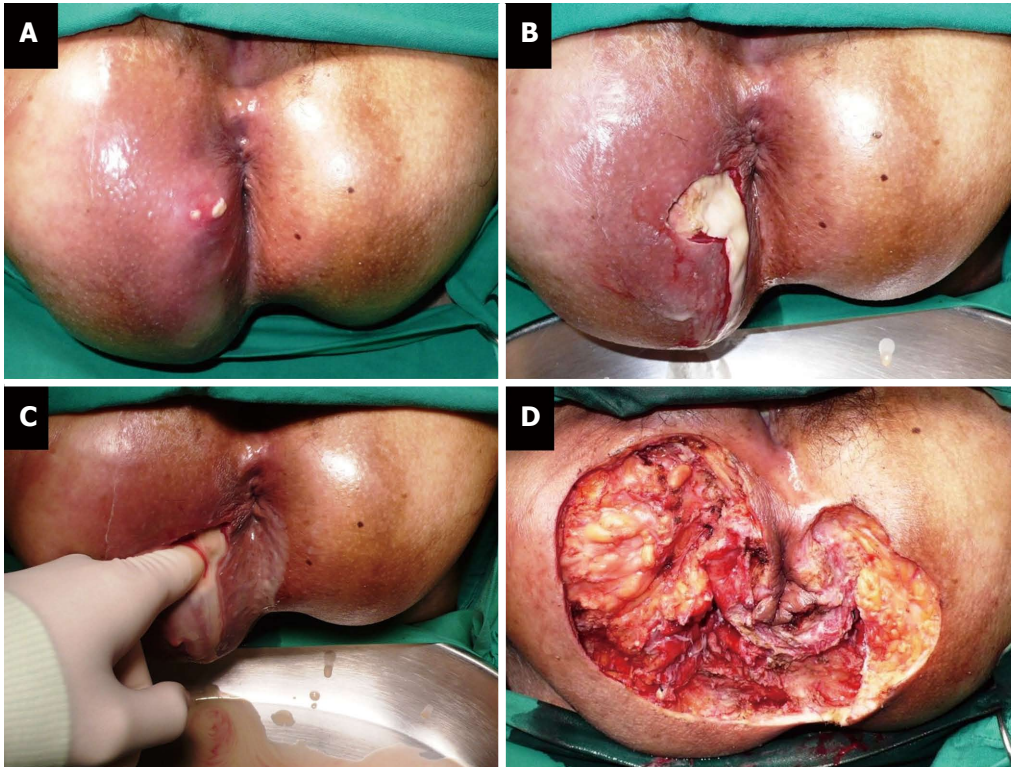


Figure 5 Perineal necrotizing fasciitis (Fournier gangrene) (A-D).

RETAINED ANORECTAL FOREIGN BODIES

Diagnosis

Diagnosis of retained foreign bodies in the rectum could pose a challenge on a clinician because patients may not give an accurate history of object insertion due to their fear and embarrassment. Many patients make some efforts to remove the object before seeking medical attention. Sexual pleasure is the most common reason for introducing foreign bodies into the rectum^[21]. However, it could be a result from an accident, trafficking of illegal drugs (body packing) or criminal assault. Therefore, attending physician must treat these patients with appropriate respect and emotional support. Foreign bodies could be sharp or blunt objects with a variety of sizes and shapes. Digital rectal examination may reveal some part of the retained foreign body. More importantly, physician must evaluate whether there is an evidence of rectal perforation or an injury to the anal sphincter. Severe pelvic pain, abdominal pain, fever, tachycardia and peritonitis are suggestive of rectal perforation. Plain abdominal radiographs may reveal the number, shape and location of retained objects as well as the presence of free air (if any). Meanwhile, ultrasonography and CT scan may help detecting non-opaque objects^[22].

Management

The goal is to successfully remove the retained foreign body without causing a further injury to bowel wall

and anal sphincter complex. This could be achieved by transanal extraction, endoscopic removal and surgical intervention (object removal *via* a colotomy by laparoscopy or laparotomy). For non-operative approach, it is wise to perform a maneuver when patients receive adequate pain control, with or without conscious sedation, in the lithotomy position^[23]. Peritonitis and failure to remove the object by means of transanal and endoscopic extraction are indications for surgery. After the removal of an object, the rectum should be assessed by endoscopic examination to detect any damage to the rectal wall - and to plan treatment accordingly. The details of various extraction techniques have been recently reviewed elsewhere^[24,25].

OBSTRUCTING RECTAL CANCER

Diagnosis

Up to 15% of patients with rectal cancer present with acute distal colonic obstruction^[26]. Marked abdominal distension, obstipation and abdominal pain are among cardinal symptoms of this situation. Vomitus with appearance and odor of feces is suggestive of long-standing obstruction. Localized peritonitis and generalized peritonitis may be seen in case of perforated rectal cancer or cecal perforation as a result of closed-loop obstruction. Obstructing rectal cancer is very likely to be a locally advanced disease but distant metastasis may be evident at the time of diagnosis^[27]. Digital rectal examination usually

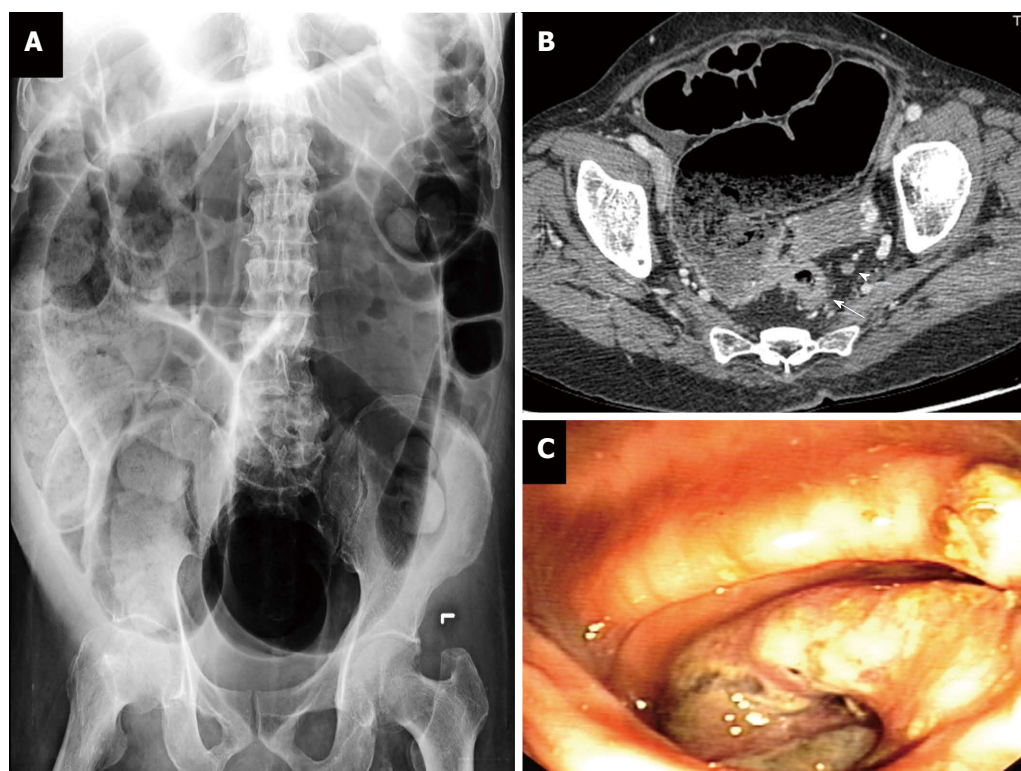


Figure 6 Obstructing rectal cancer: plain abdominal radiography (A), computerized tomography (B), and endoscopic view (C). T3 rectal cancer (white arrow) and perirectal lymph node (arrow head).

reveals rectal mass causing luminal obstruction. Plain abdominal radiography can quickly confirm mechanical colonic obstruction and determine whether there is intestinal perforation or not (Figure 6A). CT scan of chest and abdomen is very useful imaging modality for the diagnosis and evaluation of the extension of rectal cancer and its complication (Figure 6B). Differential diagnosis of obstructing rectal cancer includes rectal endometriosis, colitis cystica profunda and Crohn's disease.

Management

Initial therapy comprises intravenous fluid resuscitation, correction of metabolic derangement and intestinal decompression *via* nasogastric tube. The decision to perform surgery or endoscopic decompression for obstructing rectal cancer depended on patient's status, presence of intestinal ischemia or perforation, location and stage of rectal cancer, and surgeon or endoscopist's experience. Surgical options range from diverting colostomy to total proctocolectomy with or without restoration of bowel continuity.

For those with sign of peritonitis, intestinal ischemia or perforation, immediate surgical exploration is mandatory. Otherwise, diverting colostomy (either transverse loop colostomy or sigmoid loop colostomy) following by neoadjuvant long-course chemoradiation is recommended in patients with obstructing rectal cancer because the disease tends to be locally advanced and difficult to achieve adequate oncological

clearance in an index operation. Diverting colostomy is also an effective operation for palliation of obstructive symptoms in patients with unresectable rectal cancer or those with unresectable metastatic disease. Relieving rectal obstruction by stenting may be possible for some patients with upper or middle rectal cancer^[28].

SEXUALLY TRANSMITTED DISEASE AS ANORECTAL NON-SURGICAL EMERGENCIES

Some sexually transmitted diseases (STDs) may present in an emergency department manifesting as proctitis which could mimic other infectious proctitis and Crohn's disease. Unprotected anal receptive intercourse is the greatest risk factor for sexually transmitted proctitis. Leading symptoms of such proctitis include urgency and frequency of bowel movement, anal pain and anal discharge (mucous, purulent or bloodstained). Gonorrhea, chlamydia, herpes simplex virus, syphilis and lymphogranuloma venereum (LGV) are common STDs causing proctitis or proctocolitis^[29]. It is worth noting that *Herpes simplex virus* and *Treponema pallidum* (syphilis) breach both stratified squamous epithelium and columnar epithelium, but *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infect only columnar epithelium^[30]. *C. trachomatis* serovars D-K is less

Table 2 Diagnosis and treatment of common infectious organisms causing sexually transmitted proctitis (by the frequency of occurrence)

| Disease (causative organism) | Common symptoms and signs | Suggested investigations | Recommended first line treatment |
|---|--|---|---|
| Chlamydia (Chlamydia trachomatis serovars D-K) | Commonly asymptomatic, mild proctitis, cervicitis, vaginitis, urethritis | Nucleic acid amplification test (NAAT) from rectal, endocervical or urethral swab specimens | Azithromycin 1 g orally in a single dose OR Doxycycline 100 mg orally twice a day for 7 d |
| Gonorrhea (Neisseria gonorrhoeae) | Lower abdominal pain, diarrhea, rectal bleeding, tenesmus, purulent rectal discharge, urethral discharge and/or pharyngeal infection | Gram stain (Gram-negative diplococci) and bacterial culture from anogenital and pharyngeal swab | Ceftriaxone 250 mg IM in a single dose PLUS Azithromycin 1 g orally in a single dose |
| Herpes simplex virus (Herpes simplex virus) | Painful multiple vesicular or ulcerative lesions at perianal skin and anal canal, painful defecation, fever | Viral culture or polymerase chain reaction (PCR) from vesicular lesions | Acyclovir 400 mg orally three times a day for 7-10 d OR Acyclovir 200 mg orally five times a day for 7-10 d |
| Syphilis (Treponema pallidum) | Depending on the stage of infection - Primary syphilis: painless ulcers or chancre in the anorectal region Secondary syphilis: maculopapular rash, condyloma lata, snail-track ulcer and mucous patch at the rectum, lymphadenopathy | Darkfield examination and test to detect T. pallidum from lesion exudate or tissue | Benzathine penicillin G 2.4 million unit IM in a single dose OR Ceftriaxone 1-2 g either IV or IM for 10-14 d OR Doxycycline 100 mg orally twice a day for 14 d |
| Lymphogranuloma venereum (Chlamydia trachomatis serovars L1, L2 and L3) | Anal pain, mucous or bloody rectal discharge, anorectal ulcer, fever, inguinal or femoral lymphadenopathy | Culture, direct immunofluorescence or nucleic acid detection from rectal lesion and lymph node specimen | Doxycycline 100 mg orally twice a day for 21 d OR Erythromycin base 500 mg oral four times a day for 21 d |

virulent than *C. trachomatis* serovars L1, L2 and L3, a causative agent for LGV. As a result, LGV may present with severe proctitis, deep rectal ulcer and inguinal lymphadenopathy. Sexually transmitted proctitis in immunocompromised host e.g., HIV-infected individuals could be severe and be coinfecting with several pathogens. Table 2 summarizes the diagnosis and treatment of common infectious organisms causing sexually transmitted proctitis^[31].

ANORECTAL EMERGENCIES IN THE NEONATES

Pediatrician and pediatric surgeon should be familiar with this anorectal disorder which usually presents with delay or failure to pass meconium. In general, 99% of healthy full-term newborns will pass meconium within the first 24 h of birth^[32]. Delayed first passage of meconium raises a concern about colonic obstruction which may be related to meconium plug syndrome, Hirschsprung's disease and anorectal malformations^[33]. Notably, anorectal malformations could be associated with other congenital anomalies or as a part of combined anomalies e.g., VACTERL anomalies (Vertebral anomalies, Anorectal malformations or Anal atresia, Cardiac anomalies, TracheoEsophageal fistula or esophageal atresia, Renal and urinary anomalies, Limb lesions)^[34] or a rare complete tubular colonic duplication^[35]. Clinical examination, including

anal inspection for the presence of imperforate anus or perineal fistula, combined with plain or contrast enema abdominal radiographs would help determine the diagnosis. Some common disorders presenting as anorectal emergencies in the neonates are listed in Table 3.

EARLY POSTOPERATIVE COMPLICATIONS OF ANORECTAL SURGERY

Since many anorectal procedures can be performed safely and effectively in an ambulatory setting (day-surgery)^[36] or an overnight stay, some patients may develop complications sooner or later after hospital discharge. Common early postoperative complications that bring patients back to the hospital include acute urinary retention, bleeding, fecal impaction and anorectal sepsis.

The incidence of acute urinary retention following benign anorectal surgery ranges from 0.5%^[36] to 17%^[37] depending on the extent of surgery, anesthetic technique, analgesic method, amount of intravenous fluid given and patient's underlying disease^[38]. Bladder catheterization is the standard treatment of acute postoperative urinary retention. Should the volume of urine is less than 600 mL in low-risk individuals, the patients may be sent home without voiding^[39].

Table 3 Common anorectal disorders presenting with delay or failure to pass meconium in the neonates

| Diagnosis | Rate | Common physical findings | Suggested investigation: expected findings | Initial management |
|--|------------|--|--|---|
| Meconium plug syndrome | 1/500-1000 | Abdominal distension, normal anus and anal sphincter complex | Contrast enema radiologic examination: meconium plug in colon | Rectal stimulation with finger or saline enema |
| Hirschsprung's disease | 1/4000 | Abdominal distension, tight anal sphincter, empty rectum, sudden evacuation of stool on digital rectal examination if "transitional zone" is reached | Contrast enema radiologic examination without colonic preparation: transitional zone separating aganglionic segment and dilated proximal colon | Intravenous hydration, gastric decompression, rectal washout with warm saline, and consider colostomy in high-grade obstruction and intravenous board-spectrum antibiotics in those with suspected diagnosis of Hirschsprung-associated enterocolitis |
| Imperforate anus (IA) - Low IA = distal rectal pouch lining below or at the puborectalis muscle - High IA = distal rectal pouch lining above the puborectalis muscle | 1/5000 | Absence or stenosis of anus, perineal fistula (low IA), meconium in urine (rectourinary fistula: low or high IA), flat or not well formed median raphe (high IA), cloaca (high IA), VACTERL anomalies ¹ | Inverted lateral radiography (invertography) or transperineal ultrasonography: differentiation between low IA and high IA | Anal or fistula dilatation for temporary relief of obstruction and plan for elective posterior sagittal anorectoplasty (low IA), loop sigmoid colostomy (high IA or some low IA) |

¹VACTERL anomalies include vertebral anomalies (V), anorectal malformations (A), congenital cardiac anomalies (C), tracheoesophageal fistula or esophageal atresia (TE), renal and urinary anomalies (R), limb lesions (L).

But if the catheterized urine volume exceeds 600 mL especially in high-risk patients (e.g., severe anal pain, benign prostatic hypertrophy and immobilized patients), a self-retaining Foley catheterization may be required before discharge.

Postoperative bleeding is another common reason that brings patients to an emergency unit - with the overall incidence of 2%-4% after hemorrhoidectomy^[40] and stapled hemorrhoidopexy^[41]. A small to moderate amount of blood may be seen after an anorectal procedure especially during bowel movement and usually subsides with a conservative approach. Any major hemorrhage within the first 48 h after an operation is often caused by incomplete hemostasis. Meanwhile, delayed bleeding could be linked with a surgical site infection. Examination under anesthesia, identification and management of bleeder including re-suturing and rectal packing, correction of coagulopathy (if any) and re-hospitalization are recommended in this group of patient.

Fecal impaction after anorectal surgery is uncommon nowadays, but it could be a difficult situation to deal with especially in the early postoperative period. Postoperative fecal impaction could be a result of anal spasm, severe pain, patient's fear of defecation, inadequate intake of water and laxative, opioid-induced constipation and postsurgical edematous anorectal tissue. Mild fecal impaction may be relieved with gentle rectal enema and administration of laxatives. However, many impactions require manual disimpaction under anesthesia^[42]. The cause(s) of fecal impaction should be identified and treated accordingly.

Sepsis following an anorectal procedure is a rare but serious and potentially fatal complication. The infection could confine in anorectal region or extend

into the pelvis and retroperitoneal area^[43]. It has been described after lateral internal sphincterotomy for chronic anal fissure and after various treatment of hemorrhoid (sclerosing injection, rubber band ligation, hemorrhoidectomy and stapled hemorrhoidopexy)^[43,44]. This complication could occur in otherwise healthy patients. Unexpectedly severe and persistent pain, urinary difficulties and fever are alarming symptoms and signs of perirectal sepsis. The management of this condition is in line with that of perineal necrotizing fasciitis (Fournier gangrene) as previously discussed.

CONCLUSION

Some anorectal disorders may present as an emergency. A detailed history taking and a careful physical examination, including digital rectal examination and anoscopy, is essential for correct diagnosis and plan of treatment. Clinicians should maintain a high index of suspicion for anorectal sepsis and anorectal neoplasms. If in doubt, the attending physicians should not hesitate to consult an expert e.g., colorectal surgeon about the diagnosis, proper management and appropriate follow-up.

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2016 Gastric Cancer: Global view

HER2 heterogeneity in gastric/gastroesophageal cancers: From benchside to practice

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Abstract

HER2 is overexpressed in approximately 10%-20% of gastric and gastroesophageal junction carcinomas. In these types of cancer, accurate assessment of HER2 status is mandatory, for selecting patients who may benefit from targeted therapies with anti-HER2 drugs such as Trastuzumab. This manuscript focuses on HER2 in gastric carcinogenesis, on optimal evaluation of HER2 and on the possible causes which may contribute to inaccurate HER2 evaluation. Similarly to breast cancer HER2 evaluation, standardization of HER2 testing in gastric cancer is necessary in diagnostic practice. The three principle aspects which require consideration are: (1) the choice of sample with regards to cancer morphology - intestinal *vs* diffuse areas; (2) the choice of scoring criteria - use of HER2 scoring criteria specific for gastric cancer; and (3) the choice of HER2 evaluation methods - use of an algorithm in which both immunohistochemistry and *in situ* hybridization play a role. Problematic issues include: (1) pre-analytic variables with particular emphasis on fixation; (2) recommended methodology for HER2 assessment (immunohistochemistry *vs* *in situ* hybridization); (3) HER2 heterogeneity both within the primary tumor and between primary tumor and metastases; (4) reliability of biopsies in HER 2 evaluation; and (5) quantity of sample (FFPE blocks from surgical specimens or endoscopic biopsies) necessary for an adequate assessment.

Key words: Gastric cancer; HER2; Heterogeneity; Immunohistochemistry; FISH

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Core tip: Accurate assessment of HER2 status is mandatory in gastric/gastroesophageal cancer, for selecting patients who may benefit from targeted therapies with anti-HER2 drugs. The three principle

aspects of HER2 evaluation which require consideration are: (1) choice of sample with regards to cancer morphology; (2) choice of scoring criteria; and (3) choice of HER2 evaluation methods. Problematic issues include: (1) pre-analytic variables; (2) recommended methodology for HER2 assessment; (3) HER2 heterogeneity both within the primary tumor and between primary tumor and metastases; (4) reliability of biopsies in HER2 evaluation; and (5) quantity of sample necessary for adequate assessment.

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INTRODUCTION

Much has changed in gastric cancer (GC) treatment in the last decade as advances are being made with regards to new, tailored and integrated therapeutic approaches. Nonetheless, prognosis for GC patients still remains dismal as diagnosis is often late and, at least in Western countries, only about half of patients undergo curative resection. Even though worldwide incidence of distal GC has been slowly decreasing, it remains one of the most common causes of cancer-related deaths, with approximately 950000 new cases/year^[1] and an estimated number of deaths close to 720000. GC incidence is closely related to geographic distribution and this is mainly due to varied lifestyle characteristics, such as diet and smoking habits, as well as *Helicobacter pylori* infection^[2]. Gastroesophageal junction carcinoma (GEJC) is showing, on the other hand, a rapid rise in incidence in Western countries with a strong predilection for white males^[3].

Despite advances in cytotoxic therapies as well as various multimodality treatments, both in the neoadjuvant and adjuvant settings, survival for patients with metastatic disease remains poor, with overall survival rates of 5%-20% at 5 years^[4,5]. A relatively recent, randomized phase III trial [Trastuzumab for Gastric Cancer (ToGA)]^[6] showed improved response rate, median progression-free survival, and overall survival when the monoclonal antibody against HER2, Trastuzumab, was added to the first-line fluoropyrimidine/platinum based treatment in HER2 positive GC/GEJC. Trastuzumab and chemotherapy have since become the new standard of treatment for patients with advanced, HER2 positive, GC/GEJC. Further trials for HER2 positive cases are ongoing, using combination therapies (e.g., Trastuzumab and Bevacizumab^[7] and new molecules, such as Lapatinib^[8]).

While the predictive role of HER2 has been widely

proven, its validity as a prognostic factor in GC/GEJC is still debated, even though more recent reports favor its negative impact on prognosis^[9-12]. Contrasting results in different studies may be partly explained by: (1) different study populations with regards to ethnicity and cancer histotype; (2) use of different assays for HER2 evaluation; (3) use of variable criteria for HER2 status evaluation (older reports used HER2 breast scoring criteria); and (4) tumor heterogeneity and its impact on the type of sample tested.

HER2 in gastric carcinogenesis

The *HER2* proto-oncogene, located on chromosome 17q21^[13], encodes for a transmembrane tyrosine-kinase receptor, involved in cell proliferation and survival. Although HER2 gene amplification, with consequent HER2 protein overexpression, were identified in GC soon after their description in breast carcinoma^[14], clinical interest in HER2 remained focused on breast cancer for many years. Following the enthusiasm of ToGA trial results, HER2 has become object of great interest even though its role in gastro-esophageal carcinogenesis is still largely unexplored.

Both distal esophagus (adenocarcinoma in Barrett's esophagus) and gastric (intestinal-type adenocarcinoma) carcinogenesis rely on a multistep process in which a major role is played by longstanding inflammation with replacement of native mucosa by metaplastic epithelium. In this setting, intestinal metaplasia (IM) represents the "carcinogenic field" in which neoplasia (intra-epithelial neoplasia and invasive adenocarcinoma) can develop^[15-17]. Few studies have focused on HER-2 status in pre-neoplastic and/or pre-invasive esophageal lesions^[18-20], and these demonstrated that the rate of HER2 overexpression/amplification increases along the carcinogenetic cascade, from low grade dysplasia (LGD) to adenocarcinoma, while Barrett's esophagus metaplastic epithelium is invariably negative. These findings suggest a possible role of HER2 in the dysplasia-adenocarcinoma sequence of the esophagus. Contrasting results were published in a larger series by Hu *et al.*^[21] including 116 adenocarcinomas, 18 LGD, 15 high grade dysplasia (HGD), 34 Barrett's Esophagus (BE) as well as 81 cases of non-intestinal columnar cell metaplasia and 86 cases of esophageal squamous epithelium. HER2 amplification was detected in only one case of HGD and in 21 (18%) adenocarcinomas while all other categories were completely negative.

Even less information is available for gastric carcinogenesis. HER2 positivity was demonstrated in 12.6% of gastric HGDs in comparison to 20.2% of invasive carcinomas by analyzing both the pre-invasive and invasive component of cancer in the same patient^[22].

New insights were provided by a comprehensive and exhaustive analysis on HER2 status in the multi-step process of esophageal (non-intestinal columnar metaplasia, IM, LGD, HGD and adenocarcinoma) and gastric (antral IM, LGD, HGD and intestinal-type

adenocarcinoma) carcinogenesis^[23]. In detail, HER2 amplification was seen in 2/25 LGD, 5/25 HGD and 7/25 adenocarcinomas of the esophagus and in 1/25 LGD, 4/25 HGD and 8/25 adenocarcinomas of the stomach while native esophageal and gastric mucosa and metaplastic lesions were invariably negative. The progressive increase of HER2 amplification rate from LGD to HGD to adenocarcinoma, provides evidence of the possible early involvement of HER2 in esophageal and gastric carcinogenesis. Recent studies on the role of microRNAs (miRNAs) further reinforce the hypothesis that HER2 dysregulation is an early event in these carcinogenetic cascades^[24] in a minority of GC/GEJCs.

HER2 EVALUATION IN PRACTICE

National and International guidelines regarding HER2 testing have tried to standardize, as much as possible, HER2 testing in diagnostic practice. The three principle aspects which require consideration are: (1) the choice of sample with regards to cancer morphology; (2) the choice of scoring criteria; and (3) the choice of HER2 evaluation methods.

Sample selection with regards to cancer morphology

HER2 positive GC/GEJC are more frequently of intestinal type or mixed while diffuse type, including signet ring cell tumors, are generally HER2 negative^[25]. In mixed-type carcinomas, samples with a prevalence of intestinal-type areas should be selected when performing HER2 evaluation. Gastro-esophageal carcinomas tend to be more often HER2 positive (33%) compared to GC (21%) according to the ToGA trial and its post hoc exploratory analysis^[6,26]. These findings may be related as GEJCs are more often of intestinal-type when compared to GC^[27,28]. HER2 expression in unusual histologic subtypes is still controversial as published reports vary greatly, even with opposite findings^[29-31].

Choice of immunohistochemistry scoring criteria

Differences in HER2 staining between breast carcinoma and gastric adenocarcinoma led to a modified Immunohistochemistry (IHC) scoring system for gastric cancer (used in the ToGA trial), which was validated on 168 GC specimens with 93.5% concordance with FISH^[32]. Differently to breast cancer, GC HER2 staining does not always show complete membranous staining and so baso-lateral or lateral staining was also considered positive, as this reflects the physiological prevalence of growth factor receptors at these sites. The new scoring system also took into account the more heterogeneous staining pattern in GC and distinguished between surgical and biopsy samples. A 10% cut off was established when evaluation was performed on surgical samples whereas a single cluster of at least 5 positive cells was sufficient in endoscopic biopsies^[33]. Staining

intensity was however maintained similar to breast cancer in three categories *i.e.*, faint, moderate or intense. Such modified criteria proved to be effective in predicting response to treatment^[6] within the ToGA trial.

Choice of HER2 evaluation method

Early studies reported concordance rates between IHC and *in situ* hybridization (ISH) to be lower in GC/GEJC than breast cancer, thus suggesting that additional mechanisms, other than gene amplification, may be at the basis of protein overexpression^[34,35]. In contrast, more recent studies reported high concordance rates between IHC protein overexpression and ISH amplification with 87%-98% concordance rates^[26,29,33], when IHC score 2+, equivocal cases are excluded. For these reasons a simple algorithm has been recommended by the European Medicines Agency (EMA)^[36] (www.ema.europa.eu/docs/) which states IHC as the initial testing method and ISH only for equivocal score 2+ cases. See Figure 1.

PROBLEMS IN HER2 EVALUATION

HER2 assessment relies on optimal tissue handling^[37,38], abundant tissue for evaluation and optimal scoring criteria. Problematic issues include pre-analytic variables, the identification of the best tissue samples on which to perform HER2 testing, heterogeneity within the primary tumor and heterogeneity between primary tumor and metastases.

Impact of pre-analytic variables

Standardized tissue handling, fixation regimens and immunohistochemistry techniques are mandatory for successful and reliable HER2 assessment. Clinicians should be aware that the time from biopsy/surgery to fixation (so called cold ischemia) must be minimized (especially for biopsies which dehydrate quickly) and that this may affect HER2^[39,40]. Fixation should be exclusively based on the use of 10% neutral-buffered formalin and fixation time in formalin should be a minimum of 8 h and a maximum of 48 h; prolonged fixation may also lead to unreliable HER2 results^[41,42]. HER2 testing must be performed in quality assured laboratories with validated and standardized immunohistochemical testing kits and on freshly cut sections as pre-cut sections tend to lose their antigenicity^[43]. This last point is especially important when centralizing tissue in multicenter trials.

Which methodology: IHC, FISH, CISH or SISH?

The ToGA trial identified approximately 22% of patients who showed HER2 gene amplification at fluorescent *in situ* hybridization (FISH) but no or faint protein staining at IHC (0 or 1+). These patients did not benefit from treatment with Trastuzumab. Conversely, the highest survival advantage was seen

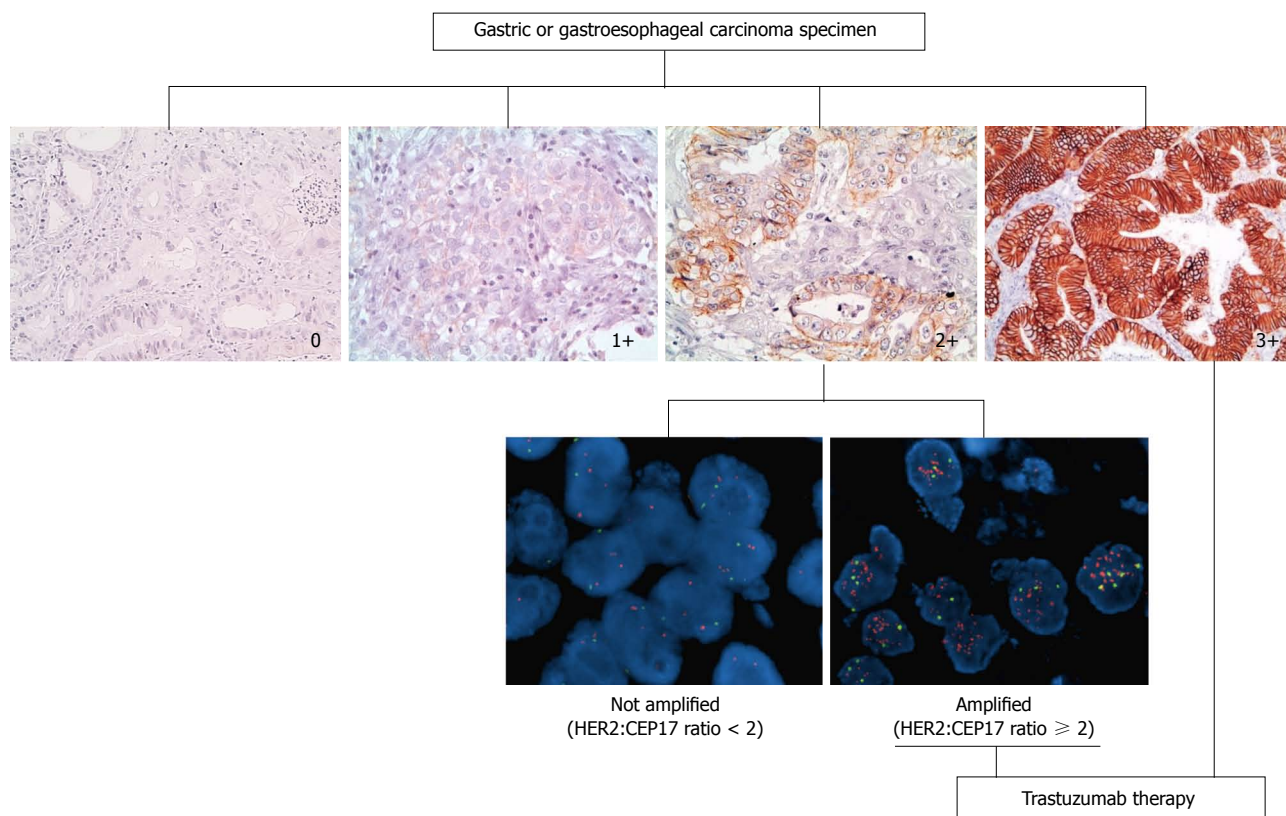


Figure 1 Diagnostic algorithm for HER2 status evaluation in gastric and gastroesophageal adenocarcinomas. Immunohistochemistry (IHC) represents the initial testing method. IHC score 0 and 1+ are considered negative while score 3+ cases are considered positive and do not need further testing. Fluorescence *in situ* hybridization testing is required only for equivocal IHC score 2+ cases. On the basis of HER2 (red spots): CEP 17 (green spots) ratio (< 2 vs ≥ 2) patients are eligible or not to anti-HER2 therapy.

in patients whose cancers were HER2 score 3+ at IHC and FISH amplified or HER2 score 2+ and FISH amplified. Differently to breast cancer, for which ISH testing may be the first approach, HER2 testing in GC should be performed by IHC as the first approach (although in the United States, the FDA approved test is indifferently by IHC or ISH). This does not mean that ISH testing is less important. Indeed, a relatively recent prospective study showed that the level of HER2 gene amplification predicts response and overall survival in HER2 positive gastric cancer treated with Trastuzumab^[44]. In close to 70 patients with advanced GC treated with Trastuzumab, the HER2/CEP17 ratio was used to predict response to treatment (optimal HER2/CEP17 ratio threshold of 4.7). ISH testing therefore, may also become useful in stratifying response rates.

Concordance studies between FISH, chromogenic (CISH) or silver based *in situ* hybridization (SISH) showed concordance rates of 91%-100%, making all these methodologies reliable for HER2 amplification testing^[45,46]. Bright field ISH techniques (CISH and SISH), may become the preferred assay in the future, as these methods enable parallel evaluation of the microscopic morphology (*i.e.*, to choose areas with intestinal morphology) as well as alignment between ISH and IHC slides.

HER2 Heterogeneity

Although initial studies reported that HER2 amplification was highly homogeneous^[47,48], tumor heterogeneity (see Figure 2) has now been shown to be extremely relevant in GC/GEJC^[49,50]. A wide range of percentages of cases showing heterogeneity appears in the Literature, from a minimum of 5% to a maximum of 69%^[22,26,32,50-52]. A possible reason for such discrepancy is that a universally accepted definition of heterogeneity is missing and, indeed, different studies applied widely different definitions. For example, Hofmann *et al.*^[32] defined heterogeneity as < 10% of tumor cells staining positive or only focal staining of tumor, Van Cutsem *et al.*^[26] identified a cut off of < 30% and Anh *et al.*^[52] defined it as a staining pattern between 10%-90% of tumor cells, leading to marked differences in heterogeneity percentages (4.8% vs 50.3% vs 68.6% respectively).

In the post hoc analysis of the HER2 screening data in the ToGA trial^[26], a comprehensive (IHC scores 1+, 2+, 3+) HER2 staining heterogeneity of about 50% was detailed and was shown to be at its greatest in the lower IHC categories (IHC score 1+ and score 2+). If assessment of heterogeneity was limited only to IHC 3+ cases, as in many other publications, percentage of heterogeneity dropped to 30%.

Another explanation for variable heterogeneity is

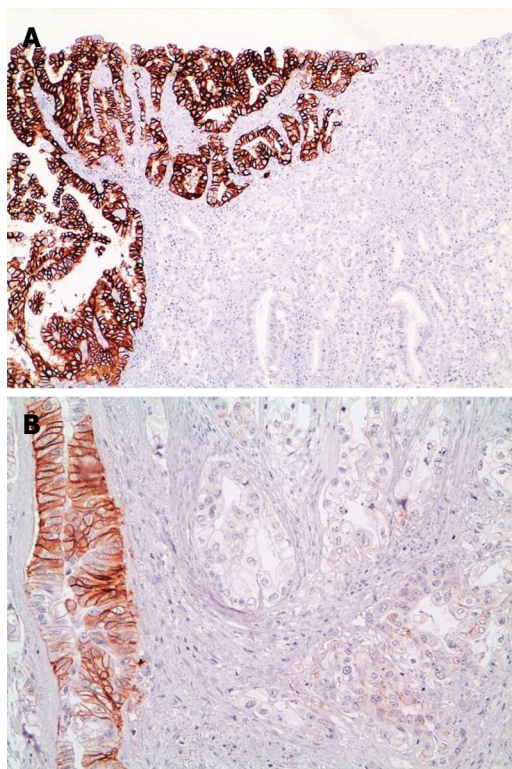


Figure 2 Heterogeneity of HER2 expression in gastric and gastroesophageal cancer. A: Gastric carcinoma, intestinal type, with HER2 overexpression (score 3+; left side) vs HER2 negativity (score 0; right side). Magnification 10 x; B: Gastroesophageal carcinoma with HER2 equivocal positivity (score 2+; left side) vs negativity (score 1+; right side). Magnification x 25.

HER2 status evaluation on tissue microarray (TMA) samples vs whole slides^[53-55], thus leading to its possible underestimation. The TMA technique performs well on homogeneously expressed proteins, however heterogeneous expression may not be correctly picked up on, even if multiple cores are used^[37].

The mechanisms leading to HER2 expression heterogeneity are still largely unknown but possibilities include neoplastic clones in which HER2 is amplified/overexpressed in an otherwise HER2 negative tumor or silencing of HER2 expression in an area of a tumor with homogeneous HER2 amplification.

Is the minimum area of > 10% cut off value appropriate in HER2 IHC assessment?

Some experts question the validity of having a minimum area of > 10% cut off value in a clearly heterogeneous expression pattern. An expert panel^[37] recommends that cases with < 10% IHC strongly stained tumor cells should also be subjected to ISH testing to reduce false-negative results, and that if amplification is detected, the case should be considered HER2 positive. This proposal has been picked up on by some international guidelines (e.g., Belgium Guidelines for HER2 testing^[56]). Furthermore, other authors suggest that a 10% cut off is subject to significant inter-observer variability^[57] and that there

is a risk of misinterpretation of the staining results leading to denial of treatment. Similarly to the modifications adopted for breast cancer HER2 testing, for which minimum area cut offs were changed from 30% to 10%^[58,59], a change in the GC HER2 staining analysis protocol may be considered in the future.

How many GC surgical resection specimen blocks should be analyzed?

Multi-block analysis^[60-64] has been shown to increase sensitivity and accuracy. False negative rates for one block analysis compared to multiblock analysis are between 7% and 10%. This is especially important if one considers that these patients, who would benefit from Trastuzumab therapy, would have been denied this chance. Laboratories should adopt a decisional workflow chart to maximize HER2 positive case discovery, taking into account costs and workload for pathologists. If one block is chosen, this must at least contain the largest amount of differentiated, intestinal type tumor, which is more likely to express HER2.

Are biopsies reliable in correctly identifying HER2 status?

With the exception of patients who have recurrent disease after surgical resection, HER2 status in inoperable patients is based on endoscopic biopsy evaluation. An important question, which stems from HER2 status heterogeneity, is whether small endoscopic biopsies are reliable for HER2 assessment. The HER2 scoring system for GC/GEJC has, in part, taken into account evaluation on biopsy tissue and, indeed, a IHC 3+ group of 5 neoplastic cells is considered sufficient to define the biopsy as HER-positive^[32,33]. A recent study by our group, analyzed^[61] a cohort of 103 matched biopsy and surgical specimens for HER2 with IHC and FISH and concordance between the two types of specimens was the main aim of the study. Eighty-nine percent of biopsies were predictive of HER2 status in surgical samples with a concordance rate of 80%, showing a high predictive value of IHC biopsy material. Most of the discordant cases were IHC HER2 negative at biopsy but showed IHC positivity and gene amplification on the surgical specimen. A probable explanation for false negative HER2 status on biopsy is heterogeneity^[52,65] whereas HER2 positivity on biopsy and not on surgical resections may be due to prolonged cold ischemia and/or over or under-fixation in larger specimens^[66]. Other studies have found variable concordance rates between biopsy and paired surgical resections ranging from 45.5% to 94%^[10,22,50,52,65,67-69] questioning the reliability of HER2 status on biopsy material. From a practical point of view, one possible option is to consider ISH analysis for both IHC score 2+ and 1+ biopsy cases, while a second approach is in the repeat assessment^[10] of endoscopic biopsies, especially if IHC 1+ or 2+ at initial biopsy as suggested by the GASTHER 1 study. A rescue rate of 8.7% has been

shown and, indeed, cases with IHC expression of IHC 1+ or 2+ at initial biopsy are 3.1 times more likely to show HER2 positivity on repeat biopsy than those with IHC 0.

How many biopsies must be taken at endoscopy?

A major problem in HER2 assessment on endoscopic biopsies is the definition of the minimum set of biopsies which the endoscopist must submit for evaluation. National Comprehensive Cancer Network guidelines recommend more than 6 samples to be taken, but this is not evidence-based. Two recent publications^[52,70] have focused on this topic. Gullo *et al.*^[70] applied virtual biopsies on digitally scanned whole slides of resected GC/GEJCs identifying five superficial samples to be the optimal biopsy set. Anh *et al.*^[52] on the other hand used a large series of paired biopsy/resection specimens and identified 4 biopsy samples of cancer as the optimal number. In a real life situation not all samples submitted by the endoscopist prove to contain assessable neoplastic cells at histologic examination. Furthermore, endoscopists should preferentially take biopsies in the lateral parts of the tumor as this area has been shown to be more frequently HER2 positive^[71] while the central part of the tumor should be avoided when macroscopically ulcerated.

Are there differences in HER2 status between primary and metastases?

Discordance in HER2 status between primary and metastatic sites has been found in breast cancer^[72] but variable data are available for GC/GEJC^[47,73].

Most studies have reported a high concordance between primary and metastases in HER2 status with a discordance rate which varies between 1% to 14%^[47,64,73-77]. Both positive (negative in primary tumor and positive in metastasis)^[64] and negative (positive in primary tumor and negative in metastasis)^[75] conversion have been described. Possible explanations for discrepancies are genetic drift or clonal selection of HER2, during neoplastic progression, or as a consequence of intratumor heterogeneity of HER2. The second hypothesis is probably more likely as heterogeneous HER2 status is often found in the primary tumor^[64] and may not be identified if tissue is limited. An inherent problem in many of these studies^[47,64,75,78] is the evaluation of HER2 status on TMA which may underestimate heterogeneity and overestimate discrepancies between primary and metastatic sites.

The previously mentioned GASTHER 1 study^[10] has shown that repeat HER2 assessment in recurrent sites may be recommended in patients with advanced GC/GEJC whose initial evaluation was HER2 negative (5.7% patients were HER2 positive on biopsy of metastases) and that these patients show similar treatment benefits with Trastuzumab as patients identified as HER2 positive at initial evaluation. In particular, liver

as site of metastasis was 5.88 times more likely to show HER2 positivity on repeat biopsy than those who had HER2 reassessment in other metastatic sites. An evaluation of cost and potential harm of repeat biopsies is necessary, however this approach may become important in selected patients.

CONCLUSION

Despite the fact that the hitherto published data are not always consistent, the following considerations can be made: (1) HER2 assessment in GC/GEJC cancer is reliable once the pre-analytical variables and technical procedures are standardized; (2) endoscopic biopsies can provide reliable HER2 status assessment when a sufficient number of samples are available; (3) IHC and ISH assessment are both reliable, but confirmation by ISH is mandatory in cases of equivocal IHC; (4) tumor heterogeneity is a major problem (but not insurmountable) which must be taken into account when selecting samples; and (5) there is relative consistency between HER2 status in the primary tumor and in distant metastases.

The guidelines and recommendations published so far provide a good basis on which to base technical procedures and diagnostic criteria.

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2016 Gastric Cancer: Global view

Function-preserving gastrectomy for gastric cancer in Japan

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Abstract

Surgery used to be the only therapy for gastric cancer, and since its ability to cure gastric cancer was the

focus of attention, less attention was paid to function-preserving surgery in gastric cancer, though it was studied for gastroduodenal ulcer. Maki *et al* developed pylorus-preserving gastrectomy for gastric ulcer in 1967. At the same time, the definition of early gastric cancer (EGC) was being considered, histopathological investigations of EGC were carried out, and the validity of modified surgery was sustained. After the development of H₂-blockers, the number of operations for gastroduodenal ulcers decreased, and the number of EGC patients increased simultaneously. As a result, the indications for pylorus-preserving gastrectomy for EGC in the middle third of the stomach extended, and various alterations were added. Since then, many kinds of function-preserving gastrectomies have been performed and studied in other fields of gastric cancer, and proximal gastrectomy, jejunal pouch interposition, segmental gastrectomy, and local resection have been performed. On the other hand, from the overall perspective, it can be said that endoscopic resection, which was launched at almost the same time, is the ultimate function-preserving surgery under the current circumstances. The current function-preserving gastrectomies that are often performed and studied are pylorus-preserving gastrectomy and proximal gastrectomy. The reasons for this are that these procedures that can be performed with systemic lymph node dissection, and they include three important elements: (1) reduction of the extent of gastrectomy; (2) preservation of the pylorus; and (3) preservation of the vagal nerve. In addition, these operations are more likely to be performed with a laparoscopic approach as minimally invasive surgery. Of the above-mentioned three elements, reduction of the extent of gastrectomy is the most important in our view. Therefore, we should try to reduce the extent of gastrectomy if curability of the gastric cancer can still be achieved. However, if we preserve a wider residual stomach in function-preserving gastrectomy, we should pay attention to the development of metachronous gastric cancer.

Key words: Early gastric cancer; Function-preserving gastrectomy; Quality of life; Laparoscopic surgery

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Core tip: Current surgical function-preserving gastrectomies include pylorus-preserving gastrectomy, proximal gastrectomy, jejunal pouch interposition, segmental gastrectomy, and local resection. The procedures that include systemic lymph node dissection and the three elements that preserve function are pylorus-preserving gastrectomy and proximal gastrectomy.

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INTRODUCTION

Standard gastrectomy is defined in the Japanese gastric cancer treatment guidelines as the resection of at least two-thirds of the stomach with a D2 lymph node dissection. Modified surgery (limited surgery) is defined as a reduced extent of gastric resection and/or lymphadenectomy compared to standard surgery and includes optional procedures that preserve the bursa, omentum, pylorus, and vagal nerve^[1]. This modified surgery was started for early gastric cancer (EGC) cases with favorable prognoses to reduce their surgical invasiveness, and it has overlapped with the indications for laparoscopic gastrectomy, which is regarded as one form of minimally invasive surgery; in fact, modified surgery has often been performed laparoscopically^[2,3]. On the other hand, new concepts such as function-preserving surgery (FPS) that preserves gastric function, which has been sacrificed in gastric cancer surgery, were generated from the perspective of patients' postoperative quality of life (QOL)^[4,5]. Namely, an operation that is performed with the intent of achieving a better postoperative condition is thought to be FPS. Although modified surgery is apt to be used synonymously for FPS, modified surgery is not always function-preserving, and FPS does not always involve a modified procedure. However, because FPS has been derived from modified surgery, most FPS methods are currently considered modified surgery. These issues are reviewed, while providing a historical perspective.

HISTORY OF FPS

In Japan, there used to be many gastroduodenal ulcer patients, and surgery was the most effective and certain therapy until the appearance of H2-blockers^[6-9]. Therefore, it was necessary to analyze gastric physiological motor function^[10,11], control of the autonomic nervous system^[12], and the dynamics of acid secretion and hormonal secretion^[13,14], and to

investigate how these changed after gastrectomy^[15-17]. The incidence rate of gastric cancer was similarly high, and too much attention was paid to the ability of surgery to cure gastric cancer, so that less attention was paid to FPS in gastric cancer, though it was studied for gastroduodenal ulcer. In particular, there was much research on the relationship between the vagus nerve and acid secretion^[18]. Maki *et al.*^[19] developed pylorus-preserving gastrectomy (PPG) for gastric ulcer in 1967. They found that pyloric motor function changed according to the distance of the transection line from the pyloric ring in canine experiments, and they advocated that the transection line should be placed 1.5 cm proximal to the pyloric ring. Meanwhile, the definition of EGC was investigated^[1,20] from the 1960s. Then, from the 1970s, histopathological investigations of EGC were carried out in an active manner^[21-23], and the validity of modified surgery was confirmed under specific indications^[24-26]. Because the number of operations for gastroduodenal ulcer decreased after the development of H2-blockers, and the number of EGC patients simultaneously increased, modified surgery for EGC was gradually started. In the late 1980s, PPG for EGC in the middle third of the stomach had come to be performed^[27]. Thereafter, many kinds of function-preserving gastrectomies (FPGs) came to be performed and studied in other fields of gastric cancer^[28-33]. During the same period of time, endoscopists took the initiative to start endoscopic mucosal resection (EMR) for EGC, and endoscopic submucosal dissection (ESD) was developed with improved instruments and techniques. Originally, radicality and QOL conflicted with each other, but FPS tried to improve postoperative QOL while maintaining radicality. After modified surgery began, the ability of FPS to preserve gastric function and physical condition was studied.

PYLORUS-PRESERVING GASTRECTOMY

Maki *et al.*^[19] decided that pylorus-preserving gastrectomy (PPG) was indicated for gastric benign disease when the distal transection line could be made 1.5 cm proximal to the pyloric ring, and the therapeutic purpose could be achieved through resection of 1/2 to 2/3 of the stomach (Figure 1). It was initially expected that PPG would decrease dumping symptoms compared to the Billroth I method, with the later advantages of reservoir function and prevention of regurgitation of bile juice^[28]. However, meal stasis was common, so that the kinetics of gastric emptying were studied, and the length of the pyloric cuff was gradually elongated^[34,35]. Suprapyloric lymph node dissection has come to be omitted to preserve the pyloric branch of the vagal nerve and right gastric vessels^[34]. Furthermore, there has been a tendency to preserve the infrapyloric vessels^[36]. Thus, from the balance between radicality and functional preservation, PPG has become a procedure that

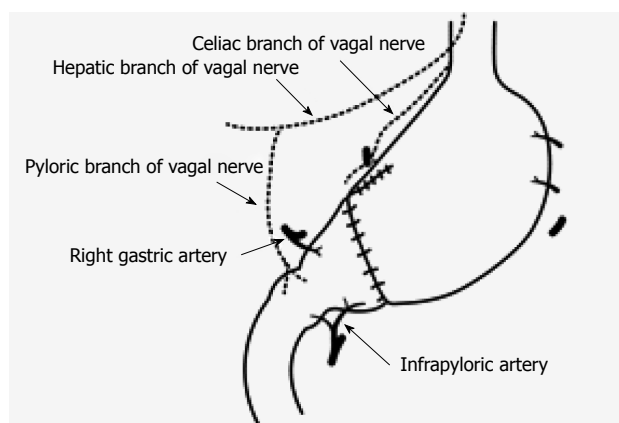


Figure 1 Pylorus-preserving gastrectomy.

preserves the upper third of the stomach and a 3 to 4-cm pyloric cuff for cN0, cT1 gastric cancer, and it preserves the hepatic branch, pyloric branch, and celiac branch as much as possible^[1]. As a result, in a large-scale postgastrectomy syndrome assessment study, Fujita *et al.*^[37] reported that better postoperative QOL was observed in PPG, including a lower incidence of diarrhea, dumping symptoms, and frequency of additional meals compared to the Billroth I procedure. Furthermore, Namikawa *et al.*^[38] reported that the size of the proximal gastric remnant significantly affected the change in body weight, scores for dissatisfaction at meals, and the dissatisfaction for daily life subscale, and preservation of a sufficient proximal gastric remnant is recommended when using PPG as FPS.

PROXIMAL GASTRECTOMY

Proximal gastrectomy (PG) began as modified surgery for gastric cancer, and Papachristou and Fortner^[39] reported that PG for adenocarcinoma of the cardia was curative only in cases of stage I and II disease (Figure 2). It turned out that the incidence rate of lymph node metastases for EGC in the upper third of the stomach was low^[29], and proximal gastrectomy is currently performed for cN0, cT1 tumors where more than half of the distal stomach can be preserved^[1]. Furthermore, the hepatic branch, pyloric branch, and celiac branch of the vagal nerve are preserved as much as possible. The reconstructive procedures need to be considered: jejunal interposition (Figure 2A), double tract (Figure 2B), and esophagogastrostomy (Figure 2C and D). The first two methods involve reconstruction with 8-15 cm of interposed jejunum between the esophagus and the remnant stomach to prevent reflux esophagitis and to observe the remnant stomach for follow-up of neoplastic tumor^[40-42]. The third method involves reconstruction by fundoplication, wrapping the remnant stomach around the circumference of the esophagus^[43] (Figure 2C) by double-flap technique, embedding the lower edge of the esophagus to the gastric submucosal layer, *etc.*^[44] (Figure 2D). For each reconstruction,

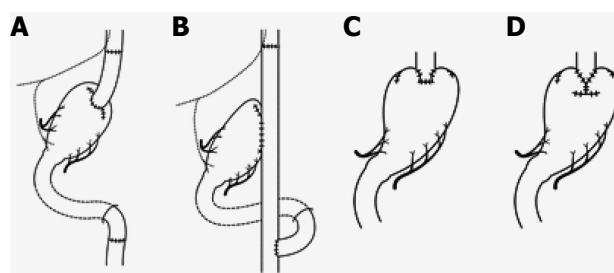


Figure 2 Proximal gastrectomy. A: Jejunal interposition; B: Double tract method; C: Esophagogastrostomy with fundoplication; D: Esophagogastrostomy with double flap technique.

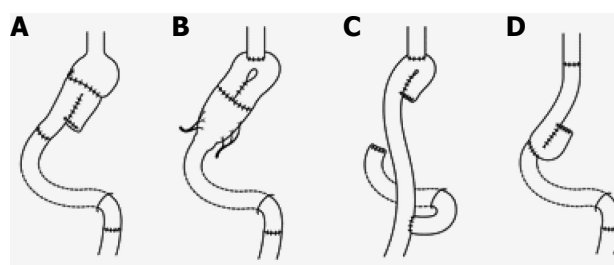


Figure 3 Jejunal pouch interposition. A: Distal gastrectomy with jejunal pouch interposition; B: Proximal gastrectomy with jejunal pouch interposition; C: Total gastrectomy with upper jejunal pouch interposition; D: Total gastrectomy with lower jejunal pouch interposition.

QOL has been evaluated. Takiguchi *et al.*^[45] reported that PG was better than total gastrectomy in terms of weight loss, necessity of additional meals, diarrhea, and dumping symptoms in a multi-institutional study. Especially in esophagogastrostomy after PG, Inada *et al.*^[46] reported that diarrhea scores and the necessity for additional meals were lower in the group with more than three-quarters of a remnant stomach compared to patients with a remnant stomach two-thirds the preoperative size. Procedures to prevent gastroesophageal reflux and the use of a pyloric bougie were considered effective ways to reduce the deterioration of QOL.

JEJUNAL POUCH INTERPOSITION

To increase the smaller gastric capacity after gastrectomy, distal gastrectomy^[30] (Figure 3A), PG^[31] (Figure 3B), and total gastrectomy^[47] (Figure 3C and D) with an interposed jejunal pouch were performed throughout Japan^[48]. Because this procedure was intended to recover the gastric reservoir function that was taken away by gastrectomy and to prevent the occurrence of reflux esophagitis, jejunal pouch interposition (JPI) was thought to be FPS. On the other hand, JPI was often added to the conventional operation (standard gastrectomy), so that it was not often a modified operation. However, Fukuhara^[49] reported that, when jejunojejunostomy was performed, disappearance of systemic intestinal peristalsis due to the division of circular muscle resulted in the occurrence of meal stasis in the jejunal pouch. Mochiki *et al.*^[50]

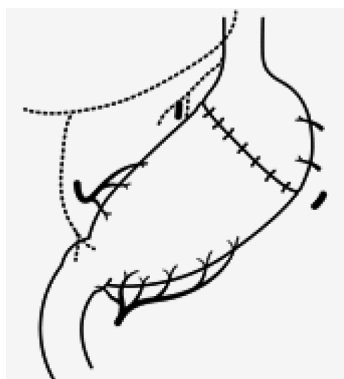


Figure 4 Segmental gastrectomy.

reported that the interposed jejunum with a pouch showed motor abnormalities. Katsube *et al.*^[51] reported a case with severe dilatation of the jejunal pouch and reflux esophagitis. Finally, Namikawa *et al.*^[52] reported that the better short-term QOL of JPI than of Roux-en-Y reconstruction decreases with time. As a result, the number of institutes performing surgery with JPI has been decreasing.

SEGMENTAL GASTRECTOMY AND LOCAL GASTRECTOMY

Segmental gastrectomy (SG) is defined as a relatively small circumferential gastric resection preserving the cardia and pylorus, excluding PPG (Figure 4). Local gastrectomy (LG) is defined as a non-circumferential gastric resection (Figure 5)^[1]. If these operative procedures could achieve radicality, they might be the ultimate FPS. Although some institutes have performed these operations under strict indications^[32,53], systemic lymph node dissection cannot be performed. Therefore, in order to assure radicality in these operations, the number of institutes that perform these operations using sentinel node navigation has been increasing^[54]. In the original concept of sentinel node navigation surgery (SNNS), detected sentinel nodes were histologically examined intraoperatively, and if no lymph node metastasis was detected, further lymphadenectomy was omitted^[55]. The feasibility and accuracy of diagnosis using sentinel node biopsy in T1 gastric cancer were evaluated in a multicenter trial (JCOG0302)^[56]. The primary endpoint was to determine the proportion of false negatives, which was defined as the number of patients with negative stained nodes by frozen section divided by those with positive stained nodes and/or positive non-stained nodes by paraffin section. It was found that the proportion of false negatives was much higher (14%) than expected (10%), and further accrual was suspended at semiannual monitoring. Thereafter, several clinical studies of lymphatic basin dissection, which is a selective lymphadenectomy to dissect stained areas, so-called lymphatic basins, containing

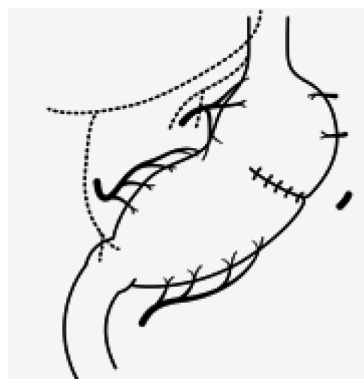


Figure 5 Local gastrectomy.

lymph nodes and lymphatic vessels stained with dye or a radioisotope or both used as a tracer for sentinel node mapping in EGC, were conducted^[57].

FUSION OF LAPAROSCOPIC SURGERY AND FUNCTION-PRESERVING SURGERY

FPS was concurrent with the beginning of laparoscopic gastrectomy and interest shifted to minimally invasive surgery. For this reason, the number of institutes where PPG^[58,59] or PG^[41,42] is performed using the laparoscopic approach has been increasing. Of course, if the efficacy of SNNS could be proven, it was thought that SG and LG would become the FPS performed under the laparoscopic approach. However, laparoscopic surgery is a kind of approach that is thoroughly minimally invasive surgery, not FPS.

ENDOSCOPIC MUCOSAL RESECTION AND ENDOSCOPIC SUBMUCOSAL DISSECTION

Generally, it may be thought that endoscopic resection is not a surgical procedure, namely FPG. However, as the techniques and instruments of endoscopic resection have developed, and its indications have expanded, the borderline between usual surgical operations and recent endoscopic resection has become unclear. Therefore, endoscopic resection was treated as an FPS in this article.

Endoscopic resection was developed as the endoscopic resection method for tumors of the colon by Rosenberg *et al.*^[60] and Deyhle *et al.*^[61] in Western countries. In Japan, there was a report of its use for gastric cancer by Hirao *et al.*^[62]. The indication for endoscopic resection was based on investigation of a large number of EGC cases who underwent open gastrectomy^[63]. Endoscopic mucosal resection (EMR) for selected intramucosal EGC cases, for which the possibility of lymph node metastasis is almost zero, has been widely accepted as a curative therapeutic strategy. The accepted indications for EMR are: (1)

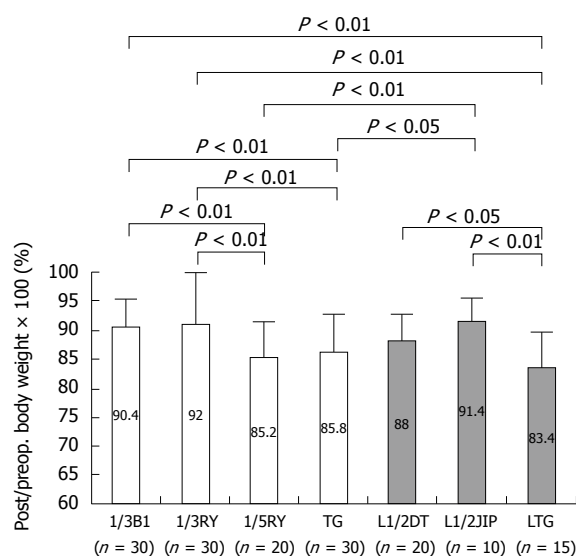


Figure 6 Postoperative to preoperative body weight ratio according to the size of the gastric remnant and the type of reconstruction following open distal and laparoscopic proximal gastrectomy including total gastrectomy. 1/3B1: Distal 2/3-gastrectomy with Billroth I reconstruction; 1/3RY: Distal 2/3-gastrectomy with Roux-en-Y reconstruction; 1/5RY: Distal 4/5-gastrectomy with Roux-en-Y reconstruction; TG: Open total gastrectomy with Roux-en-Y reconstruction; L1/2DT: Laparoscopic 1/2-proximal gastrectomy with double tract reconstruction; L1/2JIP: Laparoscopic 1/2-proximal gastrectomy with jejunal interposition reconstruction; LTG: Laparoscopic total gastrectomy with Roux-en-Y reconstruction.

well differentiated elevated lesions less than 20 mm in size; and (2) small (≤ 10 mm), depressed, well-differentiated tumors without ulceration^[1]. From further investigation of many EGC cases and the development of instruments for tissue detachment and dissection, EMR has been evolving to endoscopic submucosal dissection (ESD)^[64,65]. Currently, tumors indicated for endoscopic resection as an investigational treatment (expanded indication) are as follows: tumors clinically diagnosed as T1a and: (1) of differentiated type, UL(-), but > 2 cm in diameter; (2) of differentiated type, UL(+), and ≤ 3 cm in diameter; and (3) of undifferentiated type, UL(-), and ≤ 2 cm in diameter^[1]. After non-curative resection by EMR or ESD, additional surgical treatment should be performed; in fact, it can be said that surgical treatment could be easily added. Gastric mucosal resection as intra-gastric surgery had been performed using laparoscopic instruments through the abdominal and gastric walls^[66] and seemed to be replaced by endoscopic resection. Although the indication is restricted, endoscopic resection can be said to be the ultimate FPS with respect to reduction of invasiveness and maintenance of QOL.

EVALUATION OF FUNCTION-PRESERVING GASTRECTOMY

Given the view that function-preserving gastrectomy (FPG) preserves the autonomic nerves and maintains

physiological gastrointestinal hormonal secretion, we evaluated the postoperative physical conditions of patients who had undergone various kinds of operating methods incorporating three elements: (1) reduction of the extent of gastrectomy; (2) preservation of the pylorus; and (3) preservation of the vagal nerve^[67]. It was found that the operating methods incorporating more than two elements maintained postoperative function and QOL. In fact, PPG and PG are thought to be the ideal methods to fulfill all elements. Saito *et al.*^[68] discussed PPG and PG as FPG, and they described their oncological safety under the rigid indications and their several advantages with respect to postoperative QOL. Furthermore, in order to investigate the most important of these 3 elements, the following studies were performed. The functional outcomes of EGC patients treated by laparoscopic distal gastrectomy were compared with respect to size of the remnant stomach (1/2 vs 1/3) and the type of reconstruction (Billroth I vs Roux-en-Y). It was found that patients actually benefited from 1/2 gastrectomy rather than the typical 2/3 gastrectomy, irrespective of reconstruction method^[69]. Similar results were seen in the investigation of advanced gastric cancer patients; better functional outcomes were observed in patients with a large remnant stomach (1/3) compared to a small one (1/5), regardless of the reconstruction^[70] (Figure 6). However, a large remnant stomach sometimes shows gastric stasis, so that appropriate selection of the reconstruction method with smooth gastric emptying is needed, such as avoiding the Roux-en-Y reconstruction^[69]. Furthermore, we compared functional outcomes between different types of reconstructions (jejunal interposition method, double tract method) following open or laparoscopic 1/2- or 2/3-PG for gastric cancer. Better functional outcomes were observed in patients with a large remnant stomach and with easy flow of food into the remnant stomach regardless of whether they underwent open or laparoscopic procedures^[71]. In laparoscopic 1/2-PG with as much vagal nerve preservation as possible, the postoperative/preoperative body weight ratio was significantly higher in the jejunal interposition group in which all meals passed through the remnant stomach than in the double tract group^[41,71]. Figure 6 shows the comparison of the postoperative/preoperative body weight ratio between the open distal gastrectomy without preservation of the vagal nerve group and the laparoscopic PG with preservation of the vagal nerve group. Of the above mentioned three elements, we think that reduction of the extent of gastrectomy and passage through the stomach are the most important, although the proof for preservation of the vagal nerve is difficult. Therefore, we should try to reduce the extent of gastrectomy if curability of the gastric cancer can be achieved. However, Miwa *et al.*^[72] stated that FPG carries the risk of metachronous gastric cancer. In fact, since 1995, 160 EGC patients with negative sentinel nodes underwent FPG, which consisted of

local resection, SG, and limited distal gastrectomy. Of these 160 patients, 5 developed metachronous gastric cancer. The incidence of metachronous gastric cancer at 5 years after surgery was 2.8%, which was less than that for EMR and almost the same as that for conventional D2 distal gastrectomy^[73]. Consequently, if we could preserve a wider residual stomach as in FPG, we should pay attention to the development of metachronous gastric cancer. Specifically, regular follow-up with endoscopic examination is needed. Furthermore, for the surgeon, especially following PG, it is most important to select the reconstruction method that is appropriate for observation of the remnant stomach through endoscopy. Of course, eradication of *Helicobacter pylori* should be considered. Although the above mentioned 3 elements should be considered in FPG, further randomized, clinical trials are needed to identify the most important element.

CONCLUSION

Current surgical FPGs are thought to include PPG, PG, JPI, SG, and LG. Of these operations, the procedures that include systemic lymph node dissection and three important elements (reduction of the extent of gastrectomy, preservation of the pylorus, and preservation of the vagal nerve) are thought to be PPG and PG. Recently, the number of institutes that perform these operations with laparoscopic approaches has been increasing. Furthermore, with diagnostic examinations such as SNNS, SG and LG will become conventional as FPS in the near future.

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2016 Gastric Cancer: Global view

Exploring the role of molecular biomarkers as a potential weapon against gastric cancer: A review of the literature

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Abstract

Gastric cancer (GC) is a global health problem and a major cause of cancer-related death with high recurrence rates ranging from 25% to 40% for GC patients staging II-IV. Unfortunately, while the majority of GC patients usually present with advanced tumor stage; there is still limited evidence-based therapeutic options. Current approach to GC management consists mainly of; endoscopy followed by, gastrectomy and chemotherapy or chemo-radiotherapy. Recent studies in GC have confirmed that it is a heterogeneous disease. Many molecular characterization studies have been performed in GC. Recent discoveries of the molecular pathways underlying the disease have opened the door to more personalized treatment and better predictable outcome. The identification of molecular markers is a useful tool for clinical management in GC patients, assisting in diagnosis, evaluation of response to treatment and development of novel therapeutic modalities. While chemotherapeutic agents have certain physiological effects on the tumor cells, the prediction of the response is different from one type of tumor to the other. The specificity of molecular biomarkers is a principal feature driving their application in anticancer therapies. Here we are trying to focus on the role of molecular pathways of GC and well-established molecular markers that can guide the therapeutic management.

Key words: Gastric cancer; Molecular therapy; Targeted therapy; Biomarkers; Bioinformatics

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Core tip: We tried to highlight the role of molecular biomarkers as a predictor to chemotherapeutic response to existing regimens, aiming for better personalized therapy. Also we provided a summary of molecular markers that may aid in the development of rational therapeutic options for gastric cancer (GC) patients, aiming for improving their outcomes. However, among the plethora of agents targeting VEGF, EGFR, HER-2, IGF and mTOR pathways, trastuzumab and ramucirumab have been the only approved therapeutic options for use in advanced GC. Despite having many promising studies in their early stages, a lot have failed to prove their effectiveness in GC on the long run.

Matboli M, El-Nakeep S, Hossam N, Habieb A, Azazy AEM, Ebrahim AE, Nagy Z, Abdel-Rahman O. Exploring the role of molecular biomarkers as a potential weapon against gastric cancer: A review of the literature. *World J Gastroenterol* 2016; 22(26): 5896-5908 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i26/5896.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i26.5896>

INTRODUCTION

Gastric cancer (GC) is the second cause of cancer mortality^[1] and the fifth most common malignancy in the world, fifty percent of the cases are from Eastern Asia^[2], where China has the highest incidence^[3]. These statistics are considered to be an improvement compared to the very first estimates in 1975 where GC was the most common neoplasm^[4]. In spite of the apparent global decline in incidence and mortality estimates of GC in age-standardized figures, the absolute number of GC cases remains stable or even increasing^[5].

The five year survival rates remain disappointing despite improvements in the diagnosis and treatment of GC cases because they are usually diagnosed at an advanced stage which is rarely curable, this is in addition to the quite high rate of recurrence^[6]. Surgery has been the cornerstone in GC treatment^[7], however a high rate of intra-abdominal metastasis (80%), loco-regional (40%-80%) as well as distant (20%-40%) recurrences has been reported and luckily a survival benefit has been observed from the addition of chemotherapy or radiotherapy to surgery^[8-12].

The treatment of GC is dependent on the type of cancer tissue, the TNM staging and the general condition of the patient^[1]. Metastatic GC gives us fewer options in dealing with the disease, aiming for palliative rather than curative goal^[3].

The standard treatment options for GC include mainly: adjuvant chemotherapy or adjuvant chemo-radiation with perioperative chemotherapy. On the other hand, several recent studies evaluated the use of neoadjuvant chemotherapy solely in GC^[13], which plays a role in down-staging the disease, in addition to the eradication of any possible micrometastasis^[14-16].

While the chemotherapeutic agents that have proven effectiveness in the treatment of GC are more than a few, we cannot ignore the fact that the targeted therapy for GC is still very limited, mainly to vascular endothelial growth factor (VEGF) pathway - and HER2 - targeted agents. Recent achievements in the field of epigenetics and genetic background of the disease may enhance our chances of targeted therapeutic options in GC^[17].

On the other hand, multiple molecular biomarkers had shown their potential efficacy as diagnostic and prognostic tools in GC but they still need further validation to be used in the day-to-day clinical practice. Up till the time being, the only used markers for GC in clinical practice are carcino-embryonic antigens; CA 19-9, CA-50^[18] and CA-72^[19], which lack the high sensitivity and specificity that is needed in assessing diagnosis and prognosis of GC, making their efficacy questionable.

But as the link between the new era of the molecular markers and the treatment options is increasing; where some predict response to chemotherapy while others predict the post treatment survival or recurrence; the current review focuses on the role of molecular aberrations in affecting the therapeutic guidelines, either used for predicting the outcome of specific therapeutic agents or exploited as a therapeutic target in the tumor cells.

MOLECULAR BIOMARKERS PREDICTING THE TREATMENT RESPONSE

Prediction of chemo-resistance is an important goal in personalized medicine, where each patient is treated according to his epigenetic and genetic background. Pharmacogenomics is a rapidly growing field with hope of decreasing the burden on both the patient; by adjusting the dose, type and combination of drugs used; and the burden of cost on the healthcare system^[20,21].

Multiple genetic and epigenetic markers have been shown to have a predictive value in GC therapy, although their use is limited in the routine management of the disease, where treatment decision depends mainly on the clinical staging of the patient^[20] (Table 1).

Genetic markers

Lin *et al*^[22] compared the already published gene expression profiling signatures in GC as well as the more integrated genomic features of GC from gene

Table 1 Showing the molecular markers used in gastric cancer pharmacogenomics

| Marker type | Name | Drug predicted | Predicted drug effect |
|-----------------------|--|--|---|
| Genetic markers | 13 gene signature ^[48] | 5-FU | Sensitivity or resistance to 5-FU |
| Genetic markers | MRP4 ^[49] | Cisplatin | DDP resistance |
| Genetic markers | Metallothionein-IG and HBEGF ^[25] | Cisplatin | DDP resistance |
| Genetic markers | Dihydropyrimidine | 5-FU | 5-FU resistance |
| | Dehydrogenase and HB-EGF-like growth factor genes ^[25] | | |
| Genetic markers | Panel of genes ^[26] | Doxorubicin | Predicts response to chemotherapy |
| Genetic markers | Dihydropyrimidine | 5-FU | 5-FU resistance |
| | Dehydrogenase and HB-EGF-like growth factor genes ^[25] | | |
| Genetic markers | TP53 codon 72 polymorphism ^[50] | Paclitaxel and cisplatin | Certain genotypes predict response to combination therapy |
| lncRNA | lncRNA MRUL ^[36] | Multiple chemotherapeutic drugs | Multidrug resistance |
| Epigenetic Markers | Methylation BMP4 ^[38] | Cisplatin | High expression predicts resistance to the drug |
| Epigenetic Markers | Promoter methylation of RPRM ^[39] | CDDP and 5-FU | Prediction of response to treatment |
| Epigenetic markers | Methylation of BNIP3 and DAPK ^[37] | Fluoropyrimidine-based chemotherapy | Methylation predicts lower response to chemotherapy |
| miRNA | miRNA27a ^[34] | Fluoropyrimidine combined with oxaliplatin or paclitaxil | Prediction of response to treatment |
| MicroRNA | 58 signature mi-RNA; among them: let-7g, miR-342, miR-16, miR-181, miR-1, and miR-34 ^[33] | Cisplatin and 5-FU | Chemotherapeutic response |
| Protein markers | Thymidylate synthetase (TS) and Dihydropyrimidine dehydrogenase (DPD) ^[27,40] | 5-FU | Correlation with tumor sensitivity to 5-FU |
| Serum protein | AMBP ^[41] | paclitaxel-capecitabine | Predicts response to chemotherapy |
| Tissue protein | FOXMI ^[43] | Docetaxel | Resistance to Docetaxel |
| Transcription factor | | | |
| Protein markers | Ribosomal proteins S13 and L23 ^[47] | vincristine, adriamycin, and 5-FU | Multidrug resistance by inhibition of chemotherapy related cell death and detoxification system |
| Serum protein (ELISA) | REG4 ^[42] | 5-FU | Resistance to 5-FU containing regimens |
| Protein markers | Class III β tubulin serum level ^[45,46] | Paclitaxel plus capecitabine | Prediction of response to treatment |

MRP4: Multi drug resistance protein 4; HBEGF: Heparin-binding epidermal growth factor-like growth factor; DDP: Dihydropyrimidine; MRUL: MDR-related and upregulated lncRNA; CDDP: Cisplatin; DAPK: Death-associated protein kinase; AMBP: Alpha-1-microglobulin/bikunin precursor; foxm1: Forkhead box protein M1; REG4: Regenerating family member 4; 5-FU: 5-fluorouracil.

expression, chromosomal instability, somatic mutation, and DNA methylation. Moreover, they identified the consensus patterns across these signatures, the biological functions and the underlying molecular pathways^[22,23]. Tanaka *et al.*^[23] identified the precise prediction models of *in vitro* activity for 8 anticancer drugs (5-FU, TXL, CDDP, DOX, CPT-11, MMC, SN-38, and TXT), along with individual clinical responses to 5-FU using cDNA microarray analysis^[24].

Suganuma *et al.*^[25] reported that metallothionein-IG and heparin-binding epidermal growth factor-like growth factor (HB-EGF), glutathione-S-transferase and cyclooxygenase-2 genes were potential candidate cisplatin-resistance-related genes by oligonucleotide microarrays. For 5-FU resistance, dihydropyrimidine dehydrogenase (DPD) and HB-EGF-like growth factor genes were also suggested to be resistance-related genes^[25]. Doxorubicin response has also been linked to panel of genes including; ADAM22, CYR61, FN1, SPHK1 and GNAI1 by real-time RT-PCR in one

study^[26], but the main concern of most of these genetic signature studies is that the number of GC samples used for validation were always small in number, which means that there is a long way to go till the actual incorporation of these markers into the clinical practice.

A link was discovered between the response to cisplatin therapy and Multi-drug resistance-associated protein (MRP): when the phenotype of DDP resistance was reversed by lowering the MRP4 expression with small interfering RNA technique in GC cell line^[27].

Moreover, genetic polymorphism was linked to the response of 5-FU and cisplatin in two studies, where rs715572 and rs5754312 were linked to survival of patients treated with 5-FU + cisplatin^[28]. Also paclitaxel and cisplatin treatment response was predicted with TP53 codon 72 SNP^[29].

A group of researchers used gene expression data to describe four molecular subtypes of GC linked to disease progression and prognosis. The mesenchymal-

like type with highest recurrence frequency (63%) of the four subtypes; microsatellite-unstable tumors are hyper-mutated displaying the best overall prognosis and the lowest frequency of recurrence (22%) of the four subtypes; tumor protein 53 (TP53)-active and TP53-inactive types include patients with intermediate prognosis and recurrence rates (with respect to the other two subtypes)^[30].

Scientists proposed a molecular classification dividing GC into four subtypes: Epstein-Barr virus positive tumors with recurrent PIK3CA mutations, extreme DNA hypermethylation, and amplification of JAK2, CD274 and PDCD1LG2; microsatellite unstable tumors with elevated mutation rates, including mutations of genes encoding targetable oncogenic signaling proteins; genomically stable tumors, with mutations of RHO-family GTPase-activating proteins; and tumors with chromosomal instability with marked aneuploidy and amplification of receptor tyrosine kinases. Identification of these molecular subtypes provides an efficient roadmap for patient stratification and targeted therapies^[31].

Epigenetic markers

MicroRNA: MicroRNA was linked to the resistance to trastuzumab in one study, where it was shown that miR-21/PTEN pathway may have a regulatory effect on the treatment response^[32]. MicroRNA let-7i might predict the pathologic response to neoadjuvant chemotherapy^[29]. Also 58 signature mi-RNAs were found to predict the chemotherapeutic response of cisplatin/fluorouracil; among the apoptosis inducers are let-7g, miR-342, miR-16, miR-181, miR-1, and miR-34^[33]. miRNA-27a higher expression predicts resistance to treatment with fluoropyrimidine-containing therapy^[34].

Long non coding RNAs: Long non coding RNAs (lncRNAs) are potential biomarkers for GC especially those in blood and gastric secretions which offer a minimally invasive route^[35]. But the tissue samples are still the main site of research; where lncRNA MRUL (MDR-related and up regulated lncRNA) was associated with multi-drug chemotherapeutic resistance^[36].

Methylation related biomarkers: Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 and death associated protein kinase DAPK methylation predicts lower response to fluoropyrimidine-based chemotherapy^[37].

Decreased methylation of the Bone morphogenic protein 4 (BMP4) genes will lead to increased expression of the secreted protein, which is correlated with cisplatin resistance. BMP4 is highly expressed in cisplatin-resistant tissues and cisplatin sensitization was markedly increased with genetically targeting of BMP4 resulting in its inhibition^[38].

A study showed that increased promoter methylation will cause increased expression of Reprimo

(a highly glycosylated cellular protein) which was associated with a lower response to cisplatin and 5-FU chemotherapy, in addition the Reprimo Knockdown is associated with tumor suppression effect^[39].

Protein markers

Cellular enzymatic activity: Cellular enzymatic activity was linked to the chemotherapeutic resistance where thymidylate synthetase (TS) and DPD were associated with 5-FU tumor sensitivity^[27,40].

Cellular proteins: (1) AMBP (Alpha-1-Microglobulin/Bikunin Precursor) protein in serum was shown to predict the chemotherapeutic response to paclitaxel-capecitabine^[41]; (2) Regenerating gene family, member 4 (Reg IV or REG4) predicted resistance to 5-FU containing regimens^[42]; (3) As for tissue proteins; Forkhead Box M1 Transcription Factor (FOXM1) was shown to predict resistance to docetaxel^[43]; (4) Increased expression of β -tubulin III protein (TUBB3) in serum has been linked to taxane resistance in non small cell lung cancer^[24] and ovarian carcinoma^[44]; moreover, in a study on Chinese patients with advanced GC it was shown to predict lower response of GC to paclitaxel plus capecitabine^[45] which was confirmed later in another study^[46]; and (5) Ribosomal proteins: It was found that genetically unregulated ribosomal proteins S13 and L23 enhances vincristine, adriamycin, and 5-FU resistance by inhibition of cell death and detoxification systems induced by chemotherapy^[47].

Thus, a number of predictive biomarkers have been extensively evaluated in the setting of GC systemic therapy. However, the vast majority of these markers were derived from small scale retrospective studies; and thus, we cannot recommend incorporating any of these markers into routine practice except after careful assessment within the setting of prospective controlled clinical trials.

MOLECULAR ABERRATIONS AS POTENTIAL THERAPEUTIC TARGETS

Tumor angiogenesis inhibition

Anti-VEGFR mAbs (Ramucirumab): VEGF has long been recognized as a key regulatory pathway of angiogenesis and thus several therapeutic agents were developed to target VEGF including neutralizing antibodies to VEGF or its receptor in GC^[48-52] (Table 1). Several studies reported that the expression of VEGF and SSTR was associated with progression of GC^[53,54]. A research group used a mouse model in which VEGF-A was expressed *via* adenovirus, enabling a stromal response marked by immune infiltration and angiogenesis, and identified specific stromal gene expression signatures to discover predictive biomarkers of therapeutic response, especially to immunotherapy

and antiangiogenic agents^[55]. Ramucirumab is a fully humanized IgG1 monoclonal antibody targeting VEGFR2 thus antagonizing VEGF-A, VEGF-C and VEGF-D leading to a decrease of endothelial cell proliferation, migration and tumor vascularity, also decreasing lymphatic penetration, growth and metastasis to regional lymph node^[56,57]. Its efficacy as a second line treatment of advanced GC has been proven following the recent publication of two phase III studies (either alone or in combination with paclitaxel), and in both studies it had shown a clear overall survival benefit vs the control regimen (placebo in one study and paclitaxel monotherapy in another study)^[58,59]. Several trials are still ongoing to validate its effect in earlier stages of GC^[60].

Anti-VEGF mAbs (Bevacizumab): Bevacizumab has been evaluated for advanced GC in multiple phase II and III studies (3 phase II and 2 phase III); but unfortunately, the results were disappointingly negative in all these studies^[61-65].

Direct multi-Tyrosine kinase inhibitors (sorafenib, regorafenib, sunitinib, axitinib, dovitinib, apatinib, erlotinib, gefitinib, dacomitinib, afatinib)

Tyrosine kinase (TK) inhibition can be conducted by various drugs and most of the angiogenic factors and epidermal growth factors share a common end-pathway incorporating the TK in their receptors^[20]. Several research groups found that tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (TIE-1) and mitogen-activated protein kinase kinase 4, might serve as promising molecular biomarkers for GC prognosis. On the other hand, overexpression of TIE-1 kinase in GC patients was associated with reduced survival rates^[66-68].

Here, we give an overview on the multi-kinase TKIs which are mostly oral drugs (Table 2). Overall, they have weak to moderate activity and none of them has been approved yet in GC^[69,70].

The only successful exception of this group of agents has been Apatinib, which is a selective tyrosine kinase inhibitor against VEGF-2. A phase II trial of monotherapy in GC showed a favorable response^[71]. Later in the phase III trial, the drug showed a good safety profile and beneficial effect with improved overall survival and progression-free survival in advanced GC that was refractory to other lines of therapy^[72].

Regorafenib which is a multi-kinase inhibitor used in advanced cancers, was tested on xenograft model of patients with GC, it gave positive results regarding effectiveness for further research^[73].

Sunitinib and sorafenib are multi-kinase inhibitors (VEGF, PDGF, KIT) that have proven to be effective in a number of solid tumors, but when tested as a monotherapy or in combination in advanced GC showed a limited - if any - efficacy^[71].

Epidermal growth factor receptor inhibition based agents

EGFR is a cell surface receptor that is activated by EGF and transforming growth factor alpha. Upon activation it initiates a downstream signaling through intracellular tyrosine kinase domain resulting in DNA synthesis and cell proliferation. Among the family of EGFRs; EGFR-1 and HER-2 which are currently targets for development of drugs for GC treatment^[60]. Recent studies reported that serum HER2 levels are highly specific and demonstrated moderate diagnostic performance for HER2 tissue status in GC^[74-76].

Anti-EGFR mAbs (cetuximab/panitumumab):

EGFR is commonly over expressed in gastrointestinal malignancies. Its over expression is associated with a more aggressive phenotype and poorer survival, which suggests that EGFR may be a rational therapeutic target^[77]. Cetuximab is a humanized monoclonal antibody against EGFR and it is the most investigated in GC^[78]. Several randomized studies comparing the addition of cetuximab to conventional chemotherapies concluded that there was no clinically significant benefit associated with adding cetuximab to the conventional chemotherapies^[78].

Anti-HER-2 mAbs (Trastuzumab):

HER-2, also called ERB-2; is a tyrosine kinase receptor that when mutated exerts an oncogenic effect on cell proliferation, differentiation, programmed death and mobility; while mostly connected to breast cancer, it has proven to be the culprit in other tumors as well^[79]. HER-2 is related to increased invasiveness and metastatic potential of the tumor^[17].

HER-2 over expression has been reported in 10%-38% of GC patients^[80]. Trastuzumab is a fully humanized anti-HER-2 monoclonal antibody that is already widely accepted as a standard agent for HER-2-positive breast cancer^[81]. It is the first biological treatment to show improved survival in case of GC, immunohistochemistry score of more than +3 should receive the treatment, while > +2 should repeat the test using *in situ* hybridization^[17].

Lapatinib is another HER-2 antagonist that is used in breast cancer resistant to trastuzumab therapy, its use in GC is not supported by evidence as it did not show any activity in GC patients in many studies^[17].

PI3K/AKT/mTOR (mammalian target of rapamycin) pathway inhibitors

mTOR is active in 60% of GC cases while PI3K/Akt is active in 30% of GC cases^[82]. Intriguingly, most of the key mentioned growth factor receptors affected in GC share this pathway for signal transduction^[73], so it is expected to examine the effect of its inhibition in treatment of GC. Unfortunately, most of the inhibitors have shown low to moderate efficacy if any, despite being theoretically eligible targets, and further research

Table 2 Molecularly-targeted drugs evaluated in clinical trials for gastric cancer

| Drug name | Type | Molecular effect | Primary cancer which it is used | Effect on gastric cancer in studies |
|----------------------------------|--|--|--|--|
| Trastuzumab ^[17] | Fully humanized monoclonal antibody | Anti-HER-2 receptor protein | Breast cancer | Effective First approved molecular therapy |
| Sunitinib ^[17] | Oral multi-tyrosine kinase inhibitor | Anti-VEGF, PDGF and KIT receptors | Gastrointestinal stromal tumors, renal cell carcinoma and pancreatic neuroendocrine tumors | Limited therapeutic effect |
| Bevacizumab ^[131,132] | Fully humanized monoclonal antibody | Anti-VEGF | Colorectal cancer, non small cell lung cancer and breast cancer | Gives better survival in peritoneal metastatic disease or combined with anti-HER-2 therapy |
| Lapatinib ^[17] | Oral dual tyrosine kinase inhibitor | Anti-EGFR and HER-2 | HER-2 positive advanced breast cancer | Not effective |
| Everolimus ^[17] | Oral mTOR inhibitor | Anti-intracellular receptor FKBP12 | Renal cancer | Effective in advanced gastric cancer |
| Ramucirumab ^[17] | Fully humanized IgG1 monoclonal antibody | Anti-VEGFR-2 | Gastric and lung cancer | Effective approved |
| Cetuximab ^[17] | Monoclonal IgG antibody | Anti-EGFR | Colorectal cancer | Not effective |
| Panitumumab ^[17] | Fully humanized IgG2 monoclonal antibody | Anti-EGFR | Advanced colorectal cancer | Not effective |
| Gefitinib ^[133] | Tyrosine kinase inhibitor | Anti- EGFR | EGFR mutation positive lung cancer | Not effective |
| Matuzumab ^[134] | Fully humanized monoclonal antibody | Anti-EGFR | Not yet approved in any other indication | Moderately effective |
| Tivantinib ^[94] | Tyrosine kinase inhibitor | Selective c-Met inhibitor | Not yet approved in any other indication | moderately effective |
| Onartuzumab ^[135] | Fully humanized monoclonal antibody | Anti- extracellular domain of the tyrosine kinase receptor MET | Not yet approved in any other indication | Not effective |
| Regorafenib ^[73] | Tyrosine kinase inhibitor | Anti-angiogenic factor | Gastrointestinal stromal tumors | Found effective when tested on xenograft model with GC |
| Pembrolizumab ^[100] | Monoclonal antibody | PD-1 inhibitor | Advanced melanoma, advanced lung cancer | Promising phase IB results. Phase III results are awaited |
| Apatinib ^[72] | Tyrosine kinase inhibitor | Multikinase inhibitor | Not yet approved in any other indication | Shown to be effective in a phase III Chinese study |

VEGF: Vascular endothelial growth factor; PDGF: Platelet-derived growth factor; EGFR: Epidermal growth factor receptor; mTOR: Mammalian target of rapamycin; VEGFR: VEGF receptor; MET: Mesenchymal epithelial transition; PD-1: Programmed cell death protein-1.

is needed to determine the best drug to be used.

mTOR inhibitors including Rapamycin and its derivative everolimus have shown their effectiveness in various preclinical and early clinical trials, while their phase III trials showed disappointing outcomes^[83].

Everolimus: First introduced to GC in 2008, upon the suggestion that cisplatin-induced hypoxia will activate hypoxia inducible factor 1 alpha, and VEGF; the addition of everolimus has proven its efficacy *in vivo* in inhibiting these alternative pathways^[84].

Recently, the results of the Granite study evaluating everolimus for previously treated advanced GC have been reported and it was disappointingly negative; however, this same study showed that PIK3CA mutation and pS6 increased expression could be clues to everolimus effective salvage therapy but further prospective assessment for this point is needed^[82,85].

Rapamycin: mTOR increased expression raises the risk of recurrence by three folds, but its definite role in activation and progression of GC is not fully comprehended^[86].

Rapamycin-first known for its antifungal activity- has also anticancer activity and antiangiogenic properties. It was highly effective in preclinical trials and animal models against GC; in addition it has been shown to increase the effectiveness of chemotherapeutic drugs against GC^[83,87]. However, clear level I evidence supporting its use in GC is lacking.

Mesenchymal epithelial transition factor inhibitors

Many studies have suggested that mesenchymal epithelial transition (MET) protein was over expressed in GC patients^[88-90]. Aberrant gastric MET activation can lead to increased mesenchymal characteristics and less epithelial features, and promote cancer cell stemness, invasion, metastasis, and chemo-resistance with repressed E-cadherin; which allows tumor cells to disseminate and spread throughout the body. Stress, and hypoxia could aggravate GC *via* MET, which was significantly correlated with disease prognosis^[91].

Rilotumumab: It is a monoclonal antibody against hepatocyte growth factor receptor, thus inhibiting the MET pathway responsible for cell invasion and

proliferation^[71], it affects mainly MET-positive GC patients with good safety profile in phase II studies^[92]. Phase III studies are currently ongoing to better delineate its position in the treatment armamentarium of GC^[93].

Tivantinib and foretinib: A phase II trial to test the efficacy of tivantinib (a selective c-MET inhibitor) as a monotherapy in GC in Asian population concluded that the drug has a modest effect as a second or third choice in metastatic cases^[94]. Foretinib on the other hand; which is an oral multi-kinase inhibitor showed no benefit as a monotherapy in metastatic GC^[71].

Onartuzumab: Onartuzumab is a monoclonal antibody inhibiting the MET pathway, used mainly in advanced solid tumors either as a single agent or in addition to bevacizumab^[95], it could also be of benefit in GC; however, randomized evidence is not yet mature to support its use.

Targeting immune checkpoints

Cytotoxic T-lymphocyte antigen 4 and programmed cell death protein 1 (PD-1) are both inhibitory receptors expressed by T cells. These molecules usually appear on the surface of T cells after their activation and send an inhibitory signal^[96]. In GC, PD-1 expression on CD8+ lymphocytes is significantly higher than that of normal gastric mucosa and peripheral blood^[97]. PD-L1 overexpression, may also serve as a predictive biomarker in GC^[98].

Immunotherapy in general and immune checkpoint inhibitors - in particular - has achieved major breakthroughs in the management of a number of difficult to treat cancers like melanoma and non small cell lung cancer^[99]. For GC, a phase IB study has assessed the safety, and antitumor activity of pembrolizumab (PD-1 inhibitor) in advanced GC. Overall response rate was 32% in Asian pacific patients and 30% in rest of the world^[100]. This has lead to the launch of a number of randomized studies to further evaluate the role of this new group of agents in the management of this disease^[101].

Other pathways like insulin like growth factor-1

Insulin like growth factor (IGF)-1 gene expression may be associated with GC susceptibility and a research group showed that serum IGF-I and IGFBP-3 levels in GC patients were significantly decreased compared to the controls^[102]. But unfortunately Figitumumab; a monoclonal antibody against IGF-1; was withdrawn from phase III clinical trial of treating lung cancer due to excessive deaths, and showed no benefit in breast cancer treatment^[103]. In GC, we did not have randomized evidence supporting the use of this agent or any other agents targeting the IGF-1 pathway till now and their use should be restricted to controlled clinical studies^[104].

FUTURE CANDIDATE MOLECULAR MARKERS TO BE EXPLOITED AS CANDIDATE THERAPEUTIC TARGETS

Gastrokine 1

Certain studies suggested that Gastrokine 1 (GKN1) role in normal cells is to maintain integrity and mediate repair of gastric epithelium^[105]. Rippa *et al.*^[103] demonstrated that GKN1 mRNA present in normal gastric cells more than adenocarcinoma cells^[106]. More recent study by Xing *et al.*^[104] discovered role of GKN1 gene in GC progression, GKN1 could prevent epithelial to mesenchymal transition, decrease level of reactive oxygen species, re-expression of E-cadherin and decrease phosphatidylinositol 3-kinase (PI3K)/AKT pathway protein and so decrease metastasis in GC cell line^[107].

HDAC inhibitors

Another epigenetic mechanism that plays a role in GC is histone modifications in the form of histone post translational modifications^[108]. Histone deacetylase is thus a major target for therapeutic epigenetic inhibition.

Cancer cells are characterized by over expression of histone deacetylases (HDACs) and dysregulation of histone methyltransferases/demethylases where HDAC inhibitors have a metal binding domain that block the Zn chelation at the HDAC active sites^[109]. HDAC inhibitors represent a potential approach for cancer treatment where they act mainly through cell cycle arrest at G1 or G2-M phase together with induction of apoptosis and inhibiting angiogenesis^[110]. HDAC inhibitors are classified according to their structure into four groups: short chain fatty acids (e.g., phenylacetate and valproate), hydroxamic acids (e.g., panobinostat and belinostat), cyclicpeptides (e.g., romidepsin) and benzamide derivatives (e.g., MS-275)^[108]. HDAC inhibitors include also sodium butyrate^[111].

lncRNAs

lncRNAs play a significant role in GC progression. As lncRNAs regulate genes at different levels; namely: transcriptional, posttranscriptional and epigenetic, some of lncRNAs may have an oncogenic while other may have a tumor suppressor action^[112-115]. lncRNAs with oncogenic function show high expression in GC and its expression was correlated to TNM staging and overall survival, including HOTAIR, ANRIL, H19, GHET1, CCAT1, MALTA1, HULC and MRUL which were associated with multidrug resistance and failure of chemotherapy in GC^[116-124]. Tumor suppressor lncRNAs which were less expressed in GC cell lines include FENDRR, GAS5 and MEG3^[125-127]. lncRNAs in GC may act as competing endogenous RNA to antagonize

miRNA and relieve its repressing effect on target mRNA^[128].

Like lncRNAs; miRNA regulates gene expression at different levels^[129]. Several studies suggested that miRNA has a versatile role in GC^[115]. In an interesting study by Kim *et al.*^[133], they demonstrated a specific miRNA signature which was also related to GC chemo resistance. Also miR-610 might have a role in preventing cancer metastasis through inhibiting actin binding protein^[130].

The exact mechanism and role of lncRNAs and miRNA in GC is still unclear thus further studies are required to confirm their role in GC and consequently devising suitable therapeutic agents targeting them.

CONCLUSION

GC is a global health problem that necessitates exploiting all the available scientific advances to improve the outcome of GC patients. The use of molecular markers to predict response to GC systemic therapy has been experimented extensively. However, most of the available data were derived from small-scale retrospective analyses which do not translate to the routine use of any of these markers in day-to-day clinical practice till the time-being. On the other hand, the exploitation from our better understanding of the biology of GC has paved the way to evaluating novel agents targeting potentially carcinogenic pathways in this disease. Thus, among the plethora of agents targeting VEGF, EGFR, HER-2, IGF and mTOR pathways, only trastuzumab and ramucirumab have been approved for clinical use in advanced GC. So, while Apatinib showed impressive activity in a phase III Chinese study and may progress further to approval and pembrolizumab showed very encouraging results in early phase clinical studies, with phase III studies are ongoing, but we still have to wait to the final results because many drugs has lost the battle of approval before in GC.

We believe that properly conducted prospective randomized studies are the key to improve the outcomes of GC cases; and thus, this has to be endorsed by all scientific entities involved in GC research.

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2016 Gastrointestinal Endoscopy: Global view

Is endoscopic papillary balloon dilatation really a risk factor for post-ERCP pancreatitis?

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Abstract

Endoscopic papillary balloon dilatation (EPBD) is useful for decreasing early complications of endoscopic retrograde cholangio-pancreatography (ERCP), including bleeding, biliary infection, and perforation, but it is generally avoided in Western countries because of a relatively high reported incidence of post-ERCP pancreatitis (PEP). However, as the efficacy of endoscopic papillary large-balloon dilatation (EPLBD) becomes widely recognized, EPBD is attracting attention. Here we investigate whether EPBD is truly a risk factor for PEP, and seek safer and more effective EPBD procedures by reviewing past studies. We reviewed thirteen randomised control trials comparing EPBD and endoscopic sphincterotomy (EST) and ten studies comparing direct EPLBD and EST. Three randomized controlled trials of EPBD showed significantly higher incidence of PEP than EST, but no study of EPLBD did. Careful analysis of these studies suggested that longer and higher-pressure inflation of balloons might decrease PEP incidence. The paradoxical result that EPBD with small-calibre balloons increases PEP incidence while EPLBD does not may be due to insufficient papillary dilatation in the former. Insufficient dilatation could cause the high incidence of PEP through the use of mechanical lithotripsy and stress on the papilla at the time of stone removal. Sufficient dilation of the papilla may be useful in preventing PEP.

Key words: Endoscopic papillary balloon dilatation; Post-endoscopic retrograde cholangio-pancreatography pancreatitis; Endoscopic papillary large-balloon dilatation; Endoscopic sphincterotomy; Randomized controlled trial

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Core tip: Some recent studies suggest that Endoscopic papillary balloon dilatation (EPBD) itself does not increase post-endoscopic retrograde cholangio-pancreatography (ERCP) pancreatitis (PEP) incidence. Theoretically, endoscopic papillary large-balloon dilatation (EPLBD) can damage the papilla more than EPBD does, but even direct EPLBD without preceding sphincterotomy does not increase PEP rate. An explanation for this paradox is that procedures following EPBD, but not EPBD itself, induce PEP. Since the EPBD stress is limited around the papilla, a prophylactic pancreatic stent could protect against the damage related to EPBD. EPBD has many advantages that endoscopic sphincterotomy does not. Therefore, it is time to re-evaluate the risks and efficacy of EPBD, and to utilize it suitably instead of shelving it.

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INTRODUCTION

Staritz *et al*^[1] originally introduced endoscopic papillary balloon dilatation (EPBD) in 1983. It was developed to avoid complications of endoscopic sphincterotomy (EST) such as bleeding, perforation, and biliary infection. EPBD became popular because it was easier to perform than EST and had a possibility of preserving the function of the Oddi sphincter. However, one complication of EPBD caused anxiety: an increase in post-endoscopic retrograde cholangio-pancreatography (ERCP) pancreatitis (PEP). Many researchers studied the efficacy and safety of EPBD and found it to be feasible and acceptable, with the exception of one study. Disario *et al*^[2] performed an international multicentre study in 2004 and reported that the incidence of severe complications in the EPBD group was significantly higher than the EST group, and that two patients in the EPBD group died due to PEP. This result frightened many endoscopists, and EPBD has since been regarded as a risky procedure. EPBD has been avoided in Western countries, although it is still popular in Asian countries. On the other hand, EPBD using a large-calibre balloon (10-20 mm in diameter; endoscopic papillary large balloon dilatation; EPLBD) was recently developed for retrieving large and/or piled biliary stones, and the efficacy and safety of EPBD were also re-evaluated^[3-7].

Here, we review past studies of EPBD/EPLBD, and re-evaluate the incidence of PEP following EPBD. In addition, we discuss a safer EPBD protocol for decreasing complications.

Table 1 Pros and Cons of endoscopic papillary balloon dilatation

| | Pros | Cons |
|--|---|--|
| Endoscopic papillary balloon dilatation ¹ | Beginner-friendly Less bleeding Less perforation? Less biliary infection? Adaptive to altered anatomy Preserved sphincter function | More pancreatitis? Lower success rate of stone removal? |

¹Compared to endoscopic sphincterotomy.

REPORTED PROS AND CONS OF EPBD AND EST

The characteristics of EPBD are briefly summarized in Table 1. The pros and cons of EPBD have been often compared to those of EST. First, EPBD is technically easier and more beginner-friendly than EST. It can be adopted even in cases in which the ampulla is in a large diverticulum or cases with limited endoscopic views.

Second, EPBD has lower rates of bleeding. Indeed, one of the greatest aims of developing EPBD was to avoid post-procedural bleeding. A meta-analysis of 15 randomized clinical trials and 1768 participants showed that EPBD had significantly lower rates of bleeding than EST^[8]. Indeed, the claim of lower incidence of bleeding after EPBD is supported by most meta-analyses^[9-11]. Effects on the rates of perforation and biliary infection, however, are not consistent among the reports^[9-12].

Third, EPBD has advantages in patients with surgically altered anatomy after gastrectomy or gastric bypass surgery. In patients with Billroth II anastomoses, EPBD is associated with a significantly lower rate of bleeding, but not a higher rate of pancreatitis compared to EST^[13]. Only large biliary stone size and repeated ERCP procedures are suggested as risk factors for complications in Billroth II anastomosis cases^[14]. In patients with Roux-en-Y anastomoses, EPBD combined with balloon-assisted enteroscopy is also useful^[15].

Finally, EPBD can preserve the function of papillary sphincter even after papillary manipulation^[16]. EST destroys the function of the sphincter, often permanently. The elimination of sphincter function may allow duodenobiliary reflux and lead to recurrence of biliary stones and biliary infection. Animal studies reveal that long-lasting exposure to digestive enzymes and bacteria in the bile duct induces epithelial hyperplasia and dysplasia^[17,18]. In an animal study using live pigs, EPBD caused no architectural distortion or smooth muscle disruption, although EST caused transmural haemorrhage, smooth muscle disruption, and mucosal necrosis in the papillary structure^[19]. In a histological study of humans, EPBD mostly preserved the papillary architecture and smooth muscle^[20]. In a study using

Table 2 Endoscopic retrograde cholangio-pancreatography pancreatitis rates in endoscopic papillary balloon dilatation and endoscopic sphincterotomy in randomized control trials

| Ref. | | Study design | Year | Total patients | Significant difference from the control | | | Percentage of PEP | | Balloon size (mm) | Maximum pressure (atm) | Ballooning time (s) | Dilatation speed | Note |
|-----------------|--|--------------|------|----------------|---|------------|------------|-------------------|------|-------------------|------------------------|---------------------|------------------|-------------------|
| | | | | | Therapeutic success | ML use | PEP rate | EPBD | EST | | | | | |
| Significant | Fujita <i>et al</i> ^[33] | RCT | 2003 | 282 | - | - | EPBD > EST | 10.9 | 2.8 | 8 | Waist disappear | 15 | 3 min | |
| | Disario <i>et al</i> ^[2] | RCT | 2004 | 237 | - | - | EPBD > EST | 10.3 | 0.8 | 8 | Maximum | 60 | NM | 2 deaths in EPBD |
| | Watanabe <i>et al</i> ^[34] | RCT | 2007 | 180 | EST > EPBD | EPBD > EST | EPBD > EST | 16.7 | 6.7 | 8 | 7 | 120 | NM | |
| Non-significant | Minami <i>et al</i> ^[35] | RCT | 1995 | 40 | - | - | - | 10.0 | 10.0 | 8 | NM | 180 | NM | Manometry |
| | Bergman <i>et al</i> ^[36] | RCT | 1997 | 202 | - | EPBD > EST | - | 6.9 | 6.9 | 8 | 12 | 45-60 | 1-2 min | 1 death in EPBD |
| | Ochi <i>et al</i> ^[37] | RCT | 1999 | 110 | EST > EPBD | - | - | 0 | 3.7 | 8 | 8 | 60 × 3 times | NM | |
| | Arnold <i>et al</i> ^[38] | RCT | 2001 | 60 | EST > EPBD | NM | - | 20.0 | 10.0 | 8 | 10 | 60 × 2 times | NM | |
| | Yasuda <i>et al</i> ^[22] | RCT | 2001 | 70 | - | EPBD > EST | - | 5.7 | 5.7 | 8 | 6 | 60 × 2 times | NM | Manometry |
| | Bergman <i>et al</i> ^[13] | RCT | 2001 | 34 | - | - | - | 6.2 | 0 | 8 | 10 | 45-60 | 1-2 min | Billroth II |
| | Natsui <i>et al</i> ^[39] | RCT | 2002 | 140 | - | - | - | 5.7 | 4.3 | 8 | 8 | 120 | NM | |
| | Vlavianos <i>et al</i> ^[40] | RCT | 2003 | 202 | - | - | - | 4.8 | 1.0 | 10 | 12 | 30 | NM | |
| | Tanaka <i>et al</i> ^[41] | RCT | 2004 | 32 | - | - | - | 18.8 | 18.8 | 8 | 8 | 120 | NM | Long-term outcome |
| | Seo <i>et al</i> ^[25] | RCT | 2014 | 132 | - | - | - | 8.1 | 7.1 | 6-10 | Stone size | 90-120 | Gradually | Age < 40 yr |

RCT: Randomized control trial; ML: Mechanical lithotripsy; NM: Not mentioned.

a quantitative cholescintigraphy, hilum-duodenum transit time after EST was significantly shorter than in controls, but EPBD preserved hilum-duodenum transit time^[21]. Moreover, studies using manometry or MRI reveal that EPBD can preserve papillary function better than EST^[16,22,23]. Some studies also examined long-term outcomes. Over several years of follow-up, fewer patients develop biliary infections and recurring biliary stones after EPBD compared to EST^[24-27]. However, the effects of EPBD/EST on bile duct carcinogenesis have not been elucidated yet^[28].

In contrast to the multiple advantages of EPBD over EST, disadvantages of EPBD are few. Some studies suggest that EST is superior to EPBD in terms of success rate of stone removal. EPBD has also been associated with a higher incidence of PEP than EST. In this review we will try to elucidate whether these two disadvantages of EPBD really exist.

FACTORS IN CONVENTIONAL EPBD

An EPBD procedure consists of several variable factors: balloon size, pressure of inflation, duration of balloon dilation (ballooning time), frequency of inflation, and inflation speed. There is no standard technique dictating these factors, although guidelines for EPBD have been published^[29-32]. We summarized EPBD procedures used in past randomized controlled trials (RCTs), compared EST and conventional EPBD using

small calibre balloons, and evaluated the methods in terms of PEP rate and therapeutic efficacy (Table 2)^[2,13,22,25,33-41]. A total of 13 RCTs were included in the analysis^[2,13,22,25,33-41]. Simple comparisons of PEP rate were difficult, because they varied widely among the RCTs. Therefore, we divided the studies into two groups: one group that showed significant differences in the PEP rate between EPBD and EST (significant group)^[2,33,34], and another group that did not (non-significant group)^[13,22,25,35-41]. There was no study that showed a higher PEP rate in EST than in EPBD. Three RCTs^[2,33,34] reported that the PEP rate of EPBD was significantly higher than EST, but the remaining 10 RCTs^[13,22,25,35-41] did not show a significant difference. Three RCTs^[34,37,38] reported that the rate of therapeutic success in the first session was significantly lower in the EPBD group than in the EST group, and three other RCTs^[22,34,36] revealed that frequency of mechanical lithotripsy (ML) use in the EPBD group was higher than that in the EST group.

There was no obvious difference in EPBD procedures between the significant group^[2,33,34] and the non-significant group^[13,22,25,35-41]; however, the maximum pressure and ballooning time in the significant group tended to be lower and shorter, respectively, than in the non-significant group. Balloon size and dilatation speed were similar between the two groups. Thus, higher pressure (> 8 atm) and longer inflation (> 60 s) might be associated with lower PEP incidence in EPBD.

Table 3 Post-endoscopic retrograde cholangio-pancreatography pancreatitis rates after endoscopic papillary large-balloon dilatation without preceding endoscopic sphincterotomy

| Ref. | Study design | Year | Total patients | Significance compared to the control | | | Percentage of PEP | | Mean balloon size (mm) | Maximum pressure | Mean ballooning time (s) | Dilatation speed |
|---------------------------------------|--------------|------|----------------|--------------------------------------|--------|----------|-------------------|------------------|------------------------|------------------|--------------------------|------------------|
| | | | | Therapeutic success | ML use | PEP rate | EPLBD alone | EST alone | | | | |
| Minakari <i>et al</i> ^[42] | RCT | 2013 | 160 | - | NM | - | 11.2 | 8.7 | 15.0 | Size of stones | 60 | NM |
| Kim <i>et al</i> ^[43] | R | 2013 | 223 | - | - | - | 10.9 | 6.8 | 15.6 | Waist disappear | 38 | With caution |
| Hwang <i>et al</i> ^[44] | R | 2013 | 131 | - | - | - | 6.5 | 4.3 | 15.9 | Size of stones | 60 | Gradually |
| Li <i>et al</i> ^[45] | R | 2015 | 109 | - | - | - | 6.3 | 4.9 | 14.2 | Size of stones | 60 | Gradually |
| Oh <i>et al</i> ^[46] | RCT | 2012 | 83 | - | - | - | 5.0 | 7.0 | 11.8 | Waist disappear | 31 | Gradually |
| Omuta <i>et al</i> ^[47] | Pros | 2015 | 41 | N/A | N/A | N/A | 4.9 | N/A | 10-20 | Size of stones | 0 | Gradually |
| Kogure <i>et al</i> ^[48] | Pros | 2014 | 42 | - | - | - | 4.0 | 7.0 ¹ | 14.0 | Waist disappear | 15-60 | Gradually |
| Jeong <i>et al</i> ^[49] | R | 2009 | 38 | N/A | N/A | N/A | 2.6 | N/A | 15.5 | Waist disappear | 53 | Gradually |
| Chan <i>et al</i> ^[50] | R | 2011 | 247 | N/A | N/A | N/A | 0.8 | N/A | 13.2 | Size of stones | 282 | NM |
| Lin <i>et al</i> ^[51] | RCT | 2004 | 104 | - | - | - | 0 | 0 | 8-12 | Size of stones | 300 | NM |

¹Endoscopic papillary large-balloon dilatation (EPLBD) with preceding endoscopic sphincterotomy (EST). R: Retrospective; Pros: Prospective; RCT: Randomized controlled trial; ML: Mechanical lithotripsy; N/A: Not applicable; NM: Not mentioned.

EPLBD WITHOUT PRECEDING EST

In addition to EPBD analysis, we compared the same parameters between reported studies comparing EPLBD and EST (Table 3)^[42-51]. EPLBD following EST was excluded, because preceding EST may affect the incidence of PEP. We sorted a total of 10 studies in descending order of the PEP rate. Seven of the 10 studies compared EST groups and EPLBD with preceding EST groups^[42-46,48,51]. In all seven studies, the rates of therapeutic success, ML use, and PEP were not significantly different between the EPLBD and control groups. EPLBD without preceding EST removed large stones as easily and safely as EST. In EPLBD cases, there was no association between balloon size and PEP incidence. The dilatation speed was described as "gradual" in most studies. There were two methods to determine the maximum pressure of ballooning: one was stopping at the pressure of balloon waist disappearance, and the other was ballooning up to the size of stones. When ballooning up to the size of large stones, the waist usually disappeared before the balloon reached the target size. In the two studies^[50,51] with the lowest PEP incidence, longer (> 4 min) and higher-pressure (dilation up to the size of stones) inflation methods were adopted compared to the other eight studies^[42-49]. As with conventional EPBD with smaller calibre balloons, longer and higher-pressure inflation might also decrease PEP incidence in EPLBD.

Thus, the data imply that conventional EPBD might be associated with an increased rate of PEP, but EPLBD without preceding EST is not (Tables 2 and 3). How should we interpret these paradoxical results? In the past reports, three reasons were suggested^[4]. First, frequency of MLT use is decreased in EPLBD. Second, the patients who receive EPLBD are relatively older.

Younger age is supposed to be a risk factor of PEP^[52]. Third, EPLBD makes selective cannulation into the bile duct easier and decreases incorrect cannulation and injection into the pancreatic duct. If factors other than the balloon size are not different, EPLBD theoretically could damage the papilla more than EPBD. Therefore, it seems that papillary damage itself does not cause PEP, but rather, other procedures accompanying EPBD could cause PEP.

BALLOON SIZE, INFLATION TIME, AND INFLATION PRESSURE

Then, what is the best method of EPBD? There have been few studies evaluating the details of EPBD procedures. Concerning balloon size, Akiyama *et al*^[53] compared efficacy and safety between 10-mm-wide and 8-mm-wide balloons. The rate of complete stone removal within a single session was higher and use of lithotripsy was lower with a 10-mm-wide balloon than with an 8-mm-wide balloon. PEP and other complication rates were similar between the two balloon sizes. Li *et al*^[54] also studied the PEP rate for different balloon sizes and reported no difference. However, interpreting their study is difficult because of the small numbers of patients in each group (a total of 208 cases in five groups).

Liao *et al*^[55] studied the duration of balloon dilatation. The success rate of stone removal was higher and the PEP rate was lower with 5-min dilatation than with 1-min dilatation. In a meta-analysis reviewing randomized controlled trials, long EPBD (> 1 min) decreased not only PEP risk, but also the overall rate of complications. Conversely, short EPBD (≤ 1 min) had a higher risk of PEP than EST^[12]. Based on these

results, ESGE guidelines for prophylaxis of post-ERCP pancreatitis recommend balloon dilatation for more than 1 min^[31]. Bang *et al.*^[56] however, reported that efficacy and safety are not significantly different between 20-s and 60-s dilatations. On the other hand, Kuo *et al.*^[57] reported that papillary dilatation longer than 3 min increases the risk of recurrent biliary stones. Therefore, longer dilatation could decrease the PEP rate, but could damage the function of the papillary sphincter.

Concerning the dilatation pressure, Tsujino *et al.*^[58] compared the PEP rate in EPBD methods between one group with EPBD at 8 atm maintained for 2 min and another with the pressure at disappearance of the balloon waist maintained for 15 s. The success rate and the PEP rate were not significantly different between the two groups. There is no study on the speed of balloon dilatation, but Japanese guidelines recommend gradual dilatation just until the waist of the balloon disappears^[29].

Seo *et al.*^[59] compared the PEP rate between EPBD (retrograde dilatation) and percutaneous transhepatic papillary balloon dilatation (anterograde dilatation). The PEP rate was significantly higher after retrograde dilatation compared to antegrade dilatation. The authors considered that PEP might be associated with procedures before or after balloon dilatation, including contrast medium injection into the pancreatic duct and mechanical lithotripsy, rather than balloon dilatation itself. Lastly, Tsujino *et al.*^[60] examined risk factors for PEP after EPBD and identified only contrast medium injection into the pancreas as a risk factor.

DISCUSSION

The mechanism of EPBD-related PEP is still unclear. Damage to the pancreatic duct during papillary dilatation and papillary oedema or spasm after dilatation are potentially associated with induction of PEP. If the damage by EPBD is localized to the papilla, the placement of a prophylactic pancreatic stent could prevent EPBD-related PEP^[61]. Unfortunately, studies evaluating the efficacy of prophylactic pancreatic stents after EPBD have not been reported yet. However, the ESGE guidelines recommend placement of a prophylactic pancreatic stent when EPBD is performed, on the basis of this theoretical consideration^[31]. In addition to prophylactic pancreatic stents, endoscopic nasobiliary drainage (ENBD) attracts attention as a possible preventive measure for PEP after EPBD. ENBD has disadvantages of discomfort and cosmetic problem, and is rarely used in Western countries, although it is often used in Asian countries^[62]. Some studies show that ENBD is effective for PEP prevention after EPBD^[63-65]. It is speculated that ENBD reduces PEP rate by preventing pancreatic juice obstructions caused by residual stones or papillary oedema.

As mentioned above, the success rate of stone removal is significantly lower, and PEP rate is sig-

nificantly higher in conventional EPBD than in EST. However, complication rates are not different between EPLBD and EST. This might be because papillary dilatation by EPBD with small calibre balloons is often insufficient for stone removal, and sufficient dilatation can increase success rate and decrease PEP rate. Insufficient dilatation by EPBD may also increase the rate of mechanical lithotripsy use and may place stress on the papilla at the time of stone removal. Insufficient dilatation could lead to entrapment of residual stones at the papilla and could impair pancreatic drainage^[66,67]. Insufficient dilatation of the papilla seems to be one of the reasons for the high PEP rate in conventional EPBD. Results showing that longer and larger dilatation is better for PEP prevention also support this insight^[12,53,55]. Therefore, the papilla should be dilated to a sufficient size with enough pressure.

The maximal pressure applied in EPBD procedures may play an important role both in stone removal efficacy and safety. Among the 10 EPLBD studies in Table 3, six and four studies used the stone size^[42,44,45,47,50,51] and waist disappearance^[43,46,48,49] approaches, respectively. No significant differences in stone removal efficacy, ML use rate, and PEP rate were observed between the two approaches. Considering that EPBD with a 10-mm balloon achieved better efficacy and safety compared to that with an 8-mm balloon^[53], there is a possibility that adequate balloon size and pressure contribute to better efficacy and safety of EPBD procedures. In the waist-disappearance approach, the papilla dilatation effect with larger balloons may be greater than that with smaller balloons.

Ethnicity may have an impact on EPBD-related PEP rate. In a meta-analysis showing a higher PEP rate in EPBD groups than in EST groups, detailed analysis indicated that EPBD increased the PEP rate in Western patients ($P < 0.0001$), but not in Asian patients ($P = 0.08$)^[11]. In the study, only Western patients in the EPBD group experienced deadly pancreatitis^[2]. On the other hand, prospective studies of Asian patients show that results with EPBD and EST are both acceptable, although the PEP rate tends to be higher in the EPBD group^[9-12]. Sensitivity for EPBD-related PEP may have racial differences, just as the effects of drugs are different between different ethnic groups. In the future, endoscopic treatment procedures might be selected with consideration for the patient's racial and genetic background.

CONCLUSION

At present, EPBD is generally recognized to be a risk factor of PEP. However, some studies suggested that balloon dilatation itself does not cause PEP, but procedures accompanying insufficient dilatation of the papilla can cause PEP. The mechanism of EPBD-related PEP should be further investigated. Until then, when EPBD is performed for stone removal, it seems to be

better to dilate the papilla sufficiently (ballooning size > stone size, at least 8 mm with sufficient pressure for opening the waist; and ballooning time > 60 s) and to place a prophylactic pancreatic stent in order to prevent PEP.

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2016 Gastrointestinal Endoscopy: Global view

Recent traction methods for endoscopic submucosal dissection

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Abstract

Endoscopic mucosal resection (EMR) is problematic with regard to *en bloc* and curable resection rates.

Advancements in endoscopic techniques have enabled novel endoscopic approaches such as endoscopic submucosal dissection (ESD), which has overcome some EMR problems, and has become the standard treatment for gastrointestinal tumors. However, ESD is technically difficult. Procedure time is longer and complications such as intraoperative perforation and bleeding occur more frequently than in EMR. Recently various traction methods have been introduced to facilitate ESD procedures, such as clip with line, external forceps, clip and snare, internal traction, double scope, and magnetic anchor. Each method must be used appropriately according to the anatomical characteristics. In this review we discuss recently proposed traction methods for ESD based on the characteristics of various anatomical sites.

Key words: Endoscopic submucosal dissection; Traction; Pharyngeal cancer; Esophageal cancer; Gastric cancer; Colorectal cancer

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Core tip: Endoscopic submucosal dissection (ESD) is technically one of the most difficult endoscopic procedures. Recently, traction methods have been introduced to facilitate ESD procedures, various types of which have been proposed. Each traction method must be used appropriately according to anatomical characteristics. We discuss recently proposed traction methods for ESD based on the characteristics of various anatomical sites.

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INTRODUCTION

Endoscopic mucosal resection (EMR) has been performed for the treatment of superficial gastro-intestinal tumors since the 1980s^[1,2]. However, EMR is problematic with regard to the rates of *en bloc* and curable resection^[1]. Advancements in endoscopic techniques has led to novel endoscopic approaches such as endoscopic submucosal dissection (ESD)^[3-5], which has overcome some of the problems associated with EMR and has been applied to previously unresectable lesions, such as large tumors and tumors with ulcer scarring. Although endoscopic treatment is generally less invasive than open surgery, the technique of ESD is complicated and difficult. Because of its technical difficulty, the procedure time is longer and complications such as perforation and bleeding occur more frequently than in EMR^[6,7]. In addition, technical difficulties have prevented its widespread use, and ESD remains unpopular in Western countries^[8,9].

Recently, new concepts have been devised to facilitate the ESD procedure, one of which is the traction method^[10]. New devices and techniques have been reported for this ESD approach. Traction methods are essentially used to facilitate visualization of the submucosal layer, thus enabling accurate identification of the cutting line and submucosal vessels. Traction is thus a promising approach to help reduce the procedure time and complications, and may lead to more widespread adoption of ESD.

ESD was developed primarily as a treatment for gastric tumor, but is now also used for pharyngeal, esophageal, and colorectal tumors. Types of traction methods include clip with line, external forceps, clip and snare, internal traction, double scope, and magnetic anchor^[10]. Each traction technique has its own characteristics and must be used appropriately in accordance with individual anatomical considerations. Although Imaeda *et al.*^[10] reviewed the advantages and disadvantages of the traction method, they did not discuss its use based on anatomical features. Here, we describe in brief the various traction methods available (Table 1), followed by a discussion of recent traction techniques for ESD based on the characteristics of different anatomical sites.

CHARACTERISTICS OF EACH TRACTION METHOD

Clip-with-line method

The clip-with-line method was reported by Oyama *et al.*^[11,12] and Jeon *et al.*^[13], and is carried out as follows. A 3-0 silk line is tied to the arm part of the clip. After circumferential cutting, a clip applicator device is

inserted into the accessory channel of the endoscope, and the clip with line is mounted on the tip of the applicator. The scope is inserted again, and the clip with line is attached to the edge of the lesion. The lesion is then pulled toward the oral side using the line. Although this technique is simple, traction is directed solely through pulling (Figure 1).

External forceps method

Imaeda *et al.*^[14,15] reported the efficacy of an ESD procedure using an external grasping forceps, performed as follows. After circumferential cutting, an external grasping forceps is grasped by a second grasping forceps inserted through the accessory channel. The external forceps is delivered with the help of the second grasping forceps, taking care to avoid injuring the mucosa, especially at the esophagocardial junction. After the external grasping forceps is anchored at the edge of the lesion, the second forceps is released. The direction of traction is controlled not only by pulling but also by pushing, using the external grasping forceps (Figure 2).

Clip-and-snare method

The clip-and-snare method (CSM), which uses a hemoclip and snare, has been reported by Yasuda *et al.*^[16] and Baldaque-Silva *et al.*^[17]. The traction of this method involves pulling and pushing the lesion by a hemoclip grasped with the snare. However, delivery of the snare is sometimes difficult^[18]. Moreover, Yoshida *et al.*^[18] and Ota *et al.*^[19] reported use of the CSM using a prelooping technique (CSM-PLT), which improved delivery of the snare (Figure 3). CSM-PLT is carried out as follows. After circumferential cutting, the endoscope is withdrawn once to preloop a snare over it. The scope and snare are reinserted. A clip is inserted through the working channel of the endoscope and is used to grasp the mucosal flap. The prelooping snare is loosened and moved along the forceps up to the clip. The snare is then tightened to grasp the clip. Finally, the clip is released from its deployment device. Traction is maintained by the snare and clip independent of the scope. This method can be applied to any site.

Internal traction method

Internal traction can employ several methods, such as the medical ring, the clip band technique, and clip modifications^[20-22]. Although some differences exist among methods, internal traction is generally carried out as follows. First, a rubber band, medical ring, or nylon line is connected to the clip after circumferential cutting. Next, the clip is inserted into the working channel of the endoscope and attached to the edge of the resected lesion, then the second clip with the band is attached to the opposite mucosa. Dissection is facilitated by continuous traction exerted by this system (Figure 4).

Table 1 Characteristics of each traction method

| | Traction direction | Control of traction | Complexity of procedure | Required device |
|---|--------------------|---------------------|-------------------------|--------------------------------------|
| Clip with line method | Only pull | Possible | Simple | Requires no special device |
| External forceps method | Push and Pull | Possible | Simple | Requires no special device |
| Clip and snare methods using prelooping technique | Push and Pull | Possible | Simple | Requires no special device |
| Internal traction method | Any direction | Impossible | Complex | Requires special device |
| Double scope method | Any direction | Possible | Complex | Need for space and another endoscope |

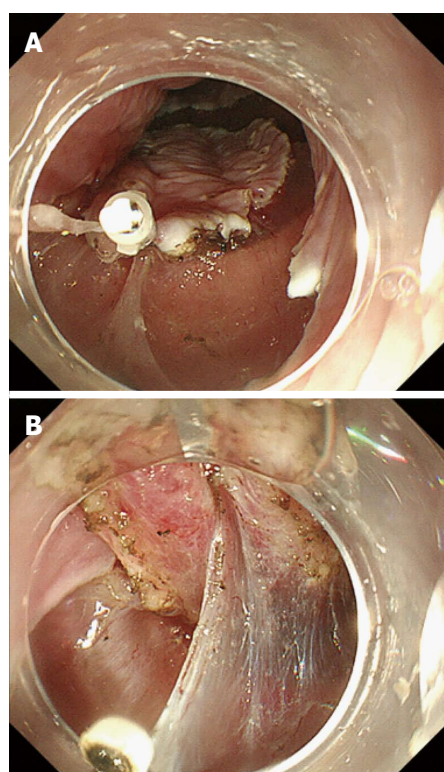


Figure 1 Clip with the line was placed on the lesion (A), and the clip and line method facilitated adequate traction (B).

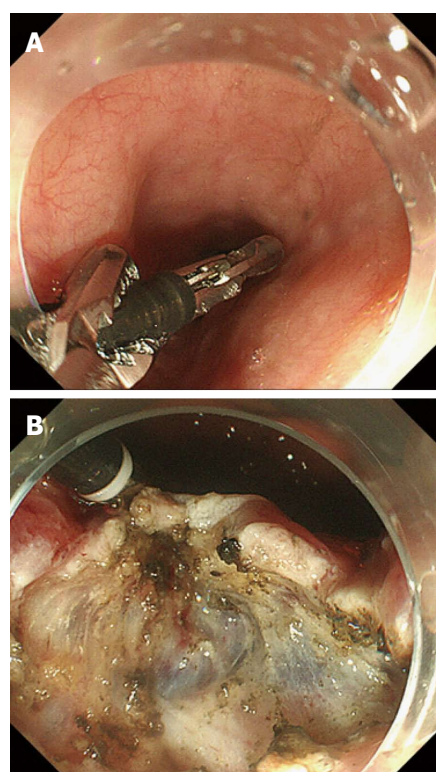


Figure 2 External grasping forceps was carefully delivered with the help of the another grasping forceps (A) and the lesion was grasped by the external grasping forceps (B).

Double-scope method

The double-scope method was reported by Morita *et al.*^[23], Higuchi *et al.*^[24], and Fujii *et al.*^[25], and is carried out as follows. Circumferential cutting is performed by the main endoscope, which is then left in the stomach after the lesion is grasped with the loop. A small-caliber endoscope is inserted along the main scope. A grasping forceps is inserted through the channel of the small-caliber endoscope and the lesion is grasped by a grasping forceps. The traction can be adjusted by the small-caliber endoscope in any direction (Figure 5).

TRACTION METHODS PERTAINING TO ANATOMICAL SITE

Head and neck

Recent advances in endoscopic devices, including magnifying endoscopy and narrow-band imaging, have enabled improvement in the early diagnosis

of superficial head and neck carcinoma^[26-28], and the use of ESD for such superficial lesions has been reported^[29,30]. Although the anatomical structure of the head and neck is generally complex, approaching the lesion using a traction device is relatively easy because of the oral proximity. Iizuka *et al.*^[31] reported ESD using Fraenkel laryngeal forceps, whose length is approximately 23 cm (Figure 6A). This method is useful to facilitate visualization of the submucosal layer because it can be adjusted in any direction as needed (Figure 6B). However, disadvantages of this method are interference between the endoscope and forceps, the need for an assistant to operate the Fraenkel laryngeal forceps, and, crucially, potential damage to the specimen. The epithelium of the pharynx is frail and easily exfoliated; histological examination of the lateral margin is unclear in some cases^[32]. Iizuka *et al.*^[31] proposed that the rear side of the specimen, instead of the edge, should be grasped by the Fraenkel

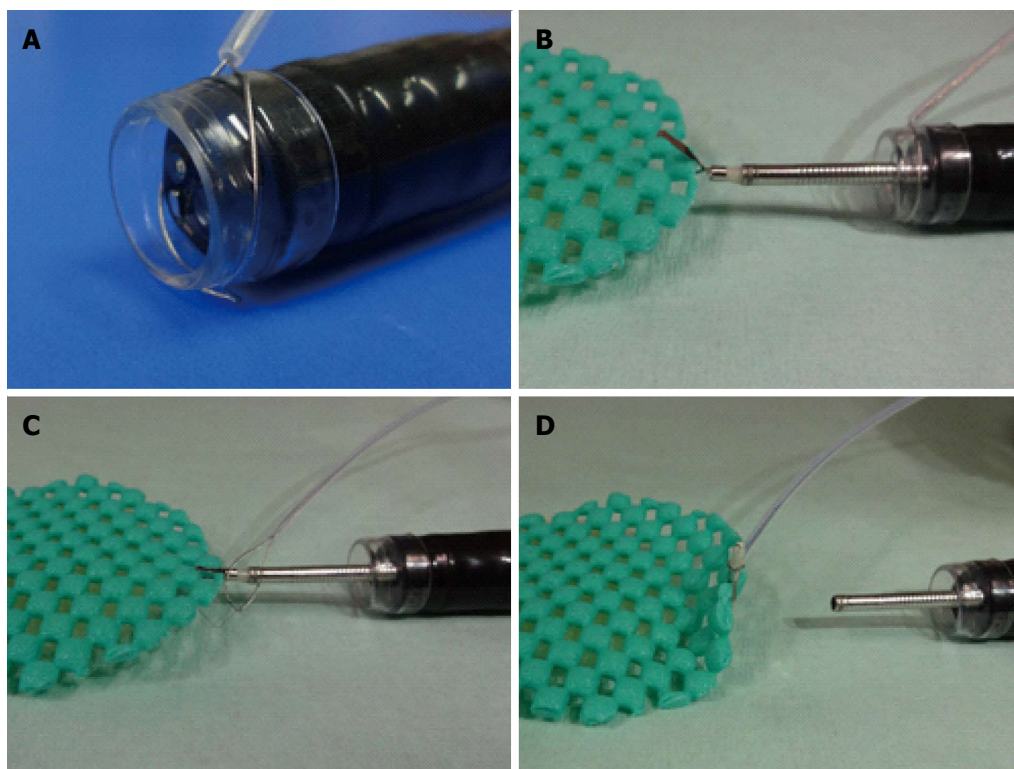


Figure 3 Schematic of our clip and snare method using a pre-looping technique. A: Endoscope with the prelooped snare; B: The clip was inserted through the working channel of the endoscope and used to grasp the mucosal flap on one side of the lesion; C: The snare, which had been pre-looped over the scope, was loosened and moved along the forceps up to the clip. We tightened the snare to grasp the clip; D: We were then able to release the clip from the forceps (from Ota *et al*^[19]).

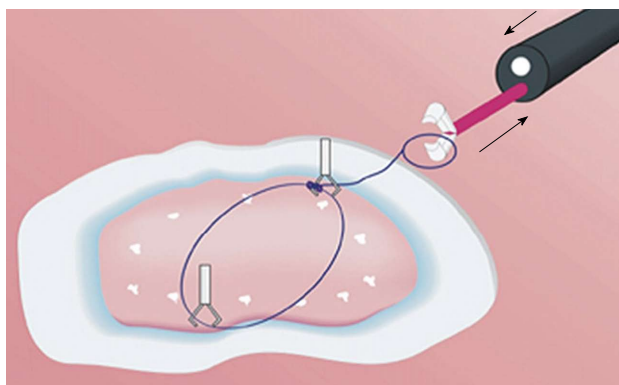


Figure 4 Schematic view of internal traction method (from Chen *et al*^[22]).

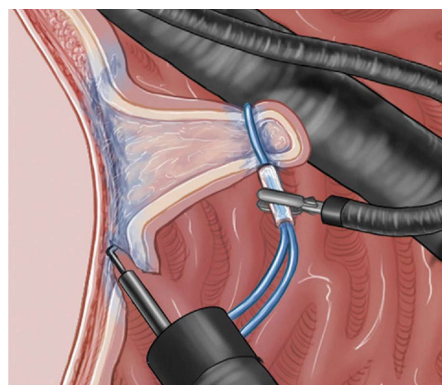


Figure 5 Schematic view of double-scope method (from Fujii *et al*^[25]).

laryngeal forceps to prevent specimen damage. Proficiency in the use of Fraenkel laryngeal forceps may be required to achieve proper traction.

Recently, endoscopic laryngopharyngeal surgery (ELPS), which was developed by modifying the ESD procedure, was reported (Figure 7)^[33,34]. ELPS is similar to ESD but less invasive^[35]. This procedure is performed by a head and neck surgeon with both hands under the guidance of a gastrointestinal scope. In a retrospective analysis, Tateya *et al*^[35] reported that the operation time (35 min) of ELPS was shorter than that of ESD (50-65.3 min). Although ELPS is becoming a major option in the treatment of superficial head

and neck carcinoma, it requires an experienced head and neck surgeon and is expensive. A prospective, randomized controlled trial is warranted to compare the functional and oncological results of ELPS and ESD.

Esophagus

The esophagus is a tube running from the pharynx to the stomach. Although its anatomical structure is not complex, the esophageal lumen is narrower than that of other organs, so most ESD procedures for esophageal tumor are performed only from the frontal view. Traction devices in the esophagus are required not to interfere with the endoscope in this narrow

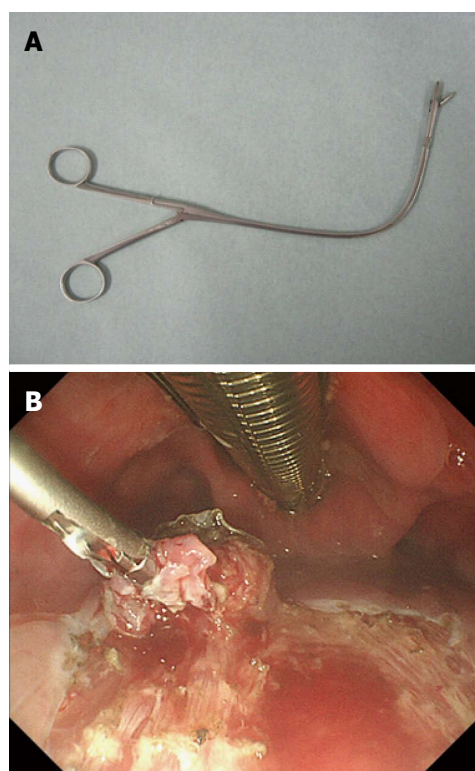


Figure 6 Fraenkel laryngeal forceps (A) and the lesion was grasped by the Fraenkel laryngeal forceps (B).

lumen. An approach such as the double-scope method is not suitable in terms of endoscope interference, while an internal traction method presents difficulty in setting two clips connected by a rubber or nylon ring because the space is limited.

The clip-with-line method was reported by Oyama *et al.*^[11,12], Ota *et al.*^[36], Jeon *et al.*^[13], and Tsao *et al.*^[37]. This technique is simple and does not require special devices and equipment^[11,12]. Although the counteraction of this method is adjusted only by pulling, it is sufficient for esophageal tumor because ESD for such a tumor is performed only from the frontal view. A prospective study was performed to confirm the safety and efficacy of this technique by Koike *et al.*^[38], who demonstrated that the dissection time (19.8 min) of the traction method was shorter than that of conventional ESD (31.8 min), without complications. Given the established safety and shorter dissection time^[38], the clip-with-line method for esophageal ESD is a viable option in the treatment of superficial esophageal tumor.

Another promising newly developed technique for esophageal ESD, reported by Ohata *et al.*^[39], Hirota *et al.*^[40], and Motohashi *et al.*^[41], is the modified external forceps method. The reported method uses an overtube equipped with a side channel or an Impact Shooter (TOP Co., Tokyo, Japan) mounted on the scope. The grasping forceps is inserted into the side channel to grasp the edge of the lesion in order to provide traction. Although this method is more

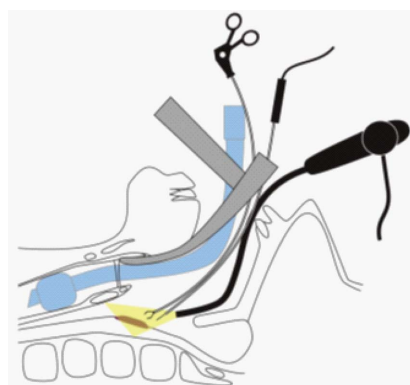


Figure 7 Schematic view and surgical setup of endoscopic laryngopharyngeal surgery (from Tateya *et al.*^[38]).

difficult than the clip-with-line method in terms of interference, the advantage is that traction can be adjusted not only by pulling but also by rotation. The feasibility and potential efficacy of this method have been demonstrated^[40,41], although the sample size was small. Further studies are warranted, including a prospective study.

Stomach

The stomach is a J-shaped organ that is distensible and may take on various shapes, and is divided into five regions: cardia, fundus, body, antrum, and pylorus. Given the idiosyncrasies of each area, the difficulty of ESD varies depending on region and/or lesion.

The clip-with-line method is useful for stomach lesions. Jeon *et al.*^[13], He *et al.*^[42], Yoshida *et al.*^[43], and Suzuki *et al.*^[44] reported that this technique is effective and safe for ESD to treat gastric neoplasms. Okamoto *et al.*^[45] reported a similar method. Like ESD for esophageal tumor, the advantage of this technique is its simplicity. This method reduced procedure time without increasing adverse events in a matched case-control study^[43,44]. Yoshida *et al.*^[43] reported that the procedure time (43 ± 24 min) in the traction group was shorter compared with that for conventional ESD (52 ± 30 min), consistent with the findings of Suzuki *et al.*^[44] (procedure time 82.2 ± 79.5 min for traction vs 118.2 ± 71.6 min for conventional ESD). A prospective, randomized controlled study with the aim of confirming the efficacy of this technique in Japan is now under way, registered in the UMIN Clinical Trial Registry as UMIN000018266. However, this technique is hampered by the fact that the direction of traction is limited. This disadvantage also applies to the pulley method, which is similar to the clip-with-line approach^[46]. Oyama *et al.*^[11] reported that the clip with line is especially useful when the cancer exists in the greater curvature of the gastric body.

The external forceps method is an option for the treatment of gastric tumor. In a retrospective analysis, Imaeda *et al.*^[14,15] reported the efficacy and safety of

ESD using an external grasping forceps for gastric neoplasia. The direction of traction was controlled not only by pulling but also by pushing using these forceps. However, disadvantages include injury to the patient and difficulty in carrying out the procedure for lesions in the cardia, lesser curvature, or posterior wall of the upper gastric body^[15].

Yasuda *et al.*^[16] and Baldaque-Silva *et al.*^[17] reported on their experience with the CSM. Traction using a snare, which is a conventional endoscopic device, enables the lesion to be pulled and pushed. As the snare is more flexible than forceps, ensuing damage is less than occurs using external forceps. Although this technique is a potential improvement on external grasping forceps, delivery of the snare is sometimes difficult and risks gastric tissue injury, especially when lesions are located in the upper body^[18]. CSM-PLT, which improved the delivery of the snare, was reported by Yoshida *et al.*^[18]. Using a prelooping technique enabled delivery of the snare to any location, and required no special device. CSM-PLT was considered effective and safe for gastric ESD. Yoshida *et al.*^[47] demonstrated that the procedure time (38.5 min) for the CSM-PLT group was shorter than that for the control group (59.5 min) in a retrospective matched-pair comparison ($P < 0.023$).

The internal traction method for gastric tumor was reported by Matsumoto *et al.*^[20], Parra-Blanco *et al.*^[21], and Chen *et al.*^[22]. However, this method also has problems. First, control of the traction direction is sometimes difficult because the traction is automatic. If the clips are incorrectly placed, traction might be applied in an incorrect direction. Parra-Blanco *et al.*^[21] recommend pushing the clip's sheath distantly to the lesion to apply the second clip to the most adequate area. Second, this method might not be applicable for lesions in the pylorus or the cardia, where space is limited^[22]. Third, this technique requires special devices and equipment.

The double-scope method is also an option for the treatment of gastric tumor^[23-25]. This technique uses a small-caliber endoscope in addition to the main scope. The traction is adjusted in any direction by the small-caliber endoscope. Maneuvering the endoscope, changing the angle, and inserting the endoscope to apply the traction are easily accomplished. However, this method has two disadvantages. First, the two endoscopes tend to interfere with each other. Second, this method is not simple. Morita *et al.*^[23] and Fujii *et al.*^[25] reported that the technique required two light sources and instruments, as well as substantial space in the endoscopy room. Although Higuchi *et al.*^[24] reported using a single light source that could be transferred between endoscopes, this technique required time and effort.

Other less reported approaches include the percutaneous traction method, the magnetic anchor method, and the robot-assisted method. von Delius *et*

al.^[48] reported a percutaneously assisted ESD using a PEG-minitrocar. However, this method is limited to the area of lesions and is more invasive than other traction methods. The magnetic anchor and robot-assisted methods have the potential to facilitate and change the procedure itself^[49-51]. However, these systems are not yet practicable in clinical practice. Further research is required for continued improvement.

Colon and rectum

The colon is long, the lumen is angulated, and the intestinal wall is thin. Colorectal ESD is consequently limited to a few high-expertise centers, thus hampering its broader application to Western countries^[52]. Moreover, colorectal ESD is not widely performed even by Eastern endoscopists because of its technical difficulty, longer procedure time, and increased risk of related complications^[53,54]. Traction methods have been attempted to facilitate the procedure of colorectal ESD. Although it is easy to apply any traction methods to the rectum, it is difficult to do so in the deep colon because of difficulty in reinserting the endoscope and adjusting the traction^[10]. It is therefore important to distinguish between the rectum and deep colon.

The double-scope method for colorectal tumors was reported by Uraoka *et al.*^[55]. This approach requires a second endoscopist to operate the thin endoscope for traction, and is limited to the rectum and distal sigmoid colon because of difficulty in inserting the thin endoscope^[55]. ESD for rectal cancers using an external forceps was reported by Imaeda *et al.*^[56]. ESD using an external forceps was possible only for rectal tumors because of difficulty in inserting and controlling the forceps^[56], making it too difficult to apply to the deep colon.

Internal traction methods such as rubber strips, S-O clips, loop-attached rubber bands, and latex bands are also promising for the treatment of colorectal tumors^[57-62]. Although control of the traction direction is difficult and a special device is required, internal traction can be advantageous for deep colon procedures. First, internal traction methods do not require reinsertion of the endoscope, as clips connected by a rubber ring or nylon are used. Clips such as the S-O clip can be passed through the instrument channel of the endoscope. Second, this system is independent, and is thus not limited by endoscopic movement. A prospective study was performed to confirm the safety and efficacy of this method by Ritsuno *et al.*^[60], who demonstrated that the procedure time for the S-O clip-assisted ESD was significantly shorter than that for conventional ESD (37.4 ± 32.6 min vs 67.1 ± 44.1 min, $P = 0.03$). Saito *et al.*^[63] reported on a similar concept, a sinker-assisted endoscopic submucosal dissection. However, this technique required reinsertion of the scope to set up the sinker, and is therefore difficult to apply to the deep colon.

Recently, two novel traction methods that do not

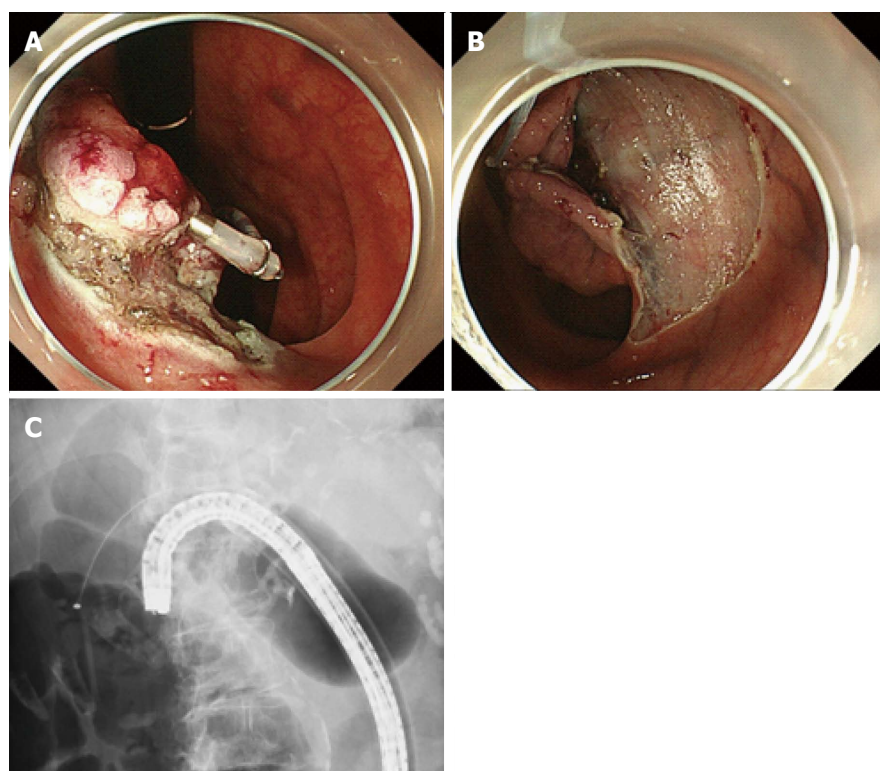


Figure 8 Clip-and-snare method enabled pushing and pulling movements for traction. A: The lesion was pulled anally in retroflexion view using a balloon overtube in the deep colon; B: Pushing the snare to facilitate visualization of submucosal layer; C: Traction is maintained by the snare and the clip independent of the scope.

Table 2 Traction method based on the anatomical site

| | Head and neck | Esophagus | Stomach | Colon and rectum |
|---|---------------------------------------|---------------------------------------|--|---|
| Clip with line method | No report, but theoretically possible | Very useful | Useful, especially in the greater curvature of gastric body | Modified method is useful in any colon |
| External forceps method | Useful | Useful | Difficult in the cardia and the lesser curvature of upper gastric body | Rectum only |
| Clip and snare methods using prelooping technique | No report, but theoretically possible | No report, but theoretically possible | Useful | Useful, but requires overtube in deep colon |
| Internal traction method | No report | No report | Difficult in the pylorus and the cardia | Useful |
| Double scope method | No report, but theoretically possible | No report | Useful | Rectum only |

require special devices or equipment and enable access to the deep colon have been reported. Yamasaki *et al.*^[64,65] reported their modified clip-with-line method, which does not require withdrawal and reinsertion of the endoscope. However, it has two disadvantages. First, it is difficult to adjust the traction. The counteraction is adjusted solely by pulling, whereby it is difficult to add further traction to tighten the line. Second, the procedural success rate of traction-assisted clip and line was 87%^[65]. Yamasaki *et al.*^[65] recommended pulling gently on the line within the proximal colon because the clip detached from three lesions, all of which were in the proximal colon.

The second method, reported by Yamada *et al.*^[66] and Ota *et al.*^[19], uses the CSM-PLT approach to deep colon endoscopy. Although this method requires

reinsertion of the endoscope, it can be applied to any colon using the prelooping technique and a balloon overtube (ST-CB1; Olympus, Tokyo). Traction is maintained by the snare and the clip independent of the scope^[19,66]. This method requires no special equipment, and is superior to the aforementioned method in two aspects. First, the clip-and-snare method enabled pushing and pulling movements for traction (Figure 8). Second, it has more flexibility. We were able to perform the ESD not only from the frontal but also the retroflex view using the clip-and-snare method. Moreover, this method enables widespread access to the submucosa through the placement of multiple clips on different edges of a lesion. This flexibility is important when ESD is performed for colorectal lesions because the clinical situation can

change from moment to moment^[19]. Yamada *et al.*^[66] demonstrated in a retrospective study that the procedure time for CSM-PLT was significantly shorter than that for a control group (45.6 min vs 70.1 min, $P = 0.047$). However, the number of patients was small. Further prospective studies are warranted to confirm the efficacy and safety of this method.

CONCLUSION

Various traction methods have been reported for ESD based on specific characteristics of each anatomical site. Each method has advantages and disadvantages, as delineated in Tables 1 and 2. Appropriate application of traction methods combined with technical proficiency will improve the outcomes of ESD procedures. Further advancements should be assessed through prospective, randomized controlled studies.

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2016 Gastrointestinal Endoscopy: Global view

Bleeding after endoscopic submucosal dissection: Risk factors and preventive methods

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Abstract

Endoscopic submucosal dissection (ESD) has become widely accepted as a standard method of treatment for superficial gastrointestinal neoplasms because it enables en block resection even for large lesions or fibrotic lesions with minimal invasiveness, and decreases the local recurrence rate. Moreover, specimens resected in an en block fashion enable accurate histological assessment. Taking these factors into consideration, ESD seems to be more advantageous than conventional endoscopic mucosal resection (EMR), but the associated risks of perioperative adverse events are higher than in EMR. Bleeding after ESD is the most frequent among these adverse events. Although post-ESD bleeding can be controlled by endoscopic hemostasis in most cases, it may lead to serious conditions including hemorrhagic shock. Even with preventive methods including administration of acid secretion inhibitors and preventive hemostasis, post-ESD bleeding cannot be completely prevented. In addition high-risk cases for post-ESD bleeding, which include cases with the use of antithrombotic agents or which require large resection, are increasing. Although there have been many reports about associated risk factors and methods of preventing post-ESD bleeding, many issues remain unsolved. Therefore, in this review, we have

overviewed risk factors and methods of preventing post-ESD bleeding from previous studies. Endoscopists should have sufficient knowledge of these risk factors and preventive methods when performing ESD.

Key words: Endoscopic submucosal dissection; Risk factor; Bleeding; Prevention; Antithrombotic agents

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Core tip: Antithrombotic agents and large resection are known to be significant risk factors for post-endoscopic submucosal dissection (post-ESD) bleeding, and as the indications for antithrombotic agents increase, and the indications for endoscopic resection are expanded, endoscopists have a chance to face an increasing number of patients with a high risk of post-ESD bleeding. Acid secretion inhibitors and preventive hemostasis are effective for the prevention of post-ESD bleeding, but do not seem to be completely effective in its prevention. Developing additional preventive methods which can reduce post-ESD bleeding more effectively will become an increasingly important issue in the future.

Kataoka Y, Tsuji Y, Sakaguchi Y, Minatsuki C, Asada-Hirayama I, Niimi K, Ono S, Kodashima S, Yamamichi N, Fujishiro M, Koike K. Bleeding after endoscopic submucosal dissection: Risk factors and preventive methods. *World J Gastroenterol* 2016; 22(26): 5927-5935 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i26/5927.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i26.5927>

INTRODUCTION

Endoscopic submucosal dissection (ESD) has become a well-established method of treatment for superficial neoplasms in the gastrointestinal tract. ESD was first developed as an advanced technique which was intended to overcome the limitations of conventional endoscopic mucosal resection (EMR) in the 1990s^[1-3]. ESD is curatively advantageous over EMR in that it enables en block fashion, regardless of tumor size, shape, ulceration or location, which contributes to the decrease in local recurrence rate. Moreover, specimens obtained by en block resection enable accurate histological diagnosis of target lesions^[4-11].

However, ESD is technically more difficult and requires a longer procedure time than EMR. In addition, ESD is accompanied by a relatively high risk of procedure-related adverse events^[4,5,7,11]. Especially, bleeding after ESD is one of the most severe adverse events because post-ESD bleeding may lead to serious conditions including hemorrhagic shock. Moreover, post-ESD bleeding can occur later than other adverse events, and may require additional treatment even

after discharge^[12-14]. Therefore, in this review article, we will focus on risk factors and preventive methods of post-ESD bleeding.

POST-ESD BLEEDING

Post-ESD bleeding, or bleeding after ESD is the most frequent adverse event associated with ESD. The incidence of bleeding after gastric ESD has been reported to range from 1.8% to 15.6%^[4,9,15-18]. On the other hand, there have been many reports that bleeding rates after esophageal or colorectal ESD are a much smaller percentages^[19-25] (Tables 1 and 2). Therefore, the reports listed in the following section are focused on the risk factors and methods of preventing bleeding after gastric ESD.

Post-ESD bleeding is generally defined as the condition that presents any clinical signs of bleeding such as hematemesis, melena, hemodynamic deterioration or downtick of > 2 g/dL in hemoglobin level and requires endoscopic hemostasis^[12,13,26].

Oda *et al* reported that 76% of post-ESD bleeding occurred within 24 h of ESD, but it can occur as late as two weeks after the procedure^[12,13,27,28]. Post-ESD bleeding can be controlled by endoscopic hemostasis in most cases (Figure 1), but it sometimes leads to life-threatening conditions that require blood transfusion or emergency surgery^[12,29]. Therefore, endoscopists should have sufficient knowledge of risk factors for this adverse event and be fully prepared for it.

RISK FACTORS

When performing gastric ESD, endoscopists should know whether their cases have a high risk of post-ESD bleeding. There have been many reports concerning the risk factors for post-ESD bleeding^[9,12-15,26,27,29-32]. Although other factors are still controversial, several studies have revealed that antithrombotic agents and resection size are significant risk factors for post-ESD bleeding^[9,14,15,26,27,29,30,33].

Antithrombotic agents

Because the number of patients taking antithrombotic agents has been increasing worldwide^[34,35], there will be an increasing necessity to perform ESD for these patients in the future. Endoscopists should pay attention to both the risks of bleeding and thromboembolism when performing ESD in this situation.

Tentative guidelines concerning the continuation and cessation of antithrombotic agents during endoscopy have been published from several societies including the Japan Gastroenterological Endoscopy Society, American Society for Gastrointestinal Endoscopy, and European Society of Gastrointestinal Endoscopy^[36-38]. ESD for patients taking antithrombotic agents is performed according to these guidelines, but currently data supporting ESD under these guidelines is

Table 1 Previous reports of bleeding after gastric endoscopic submucosal dissection

| Ref. | Organ | Year | Case No. | Post-ESD bleeding | Perforation | En block resection |
|--|---------|------|----------|-------------------|-------------|--------------------|
| Ichiro <i>et al</i> ^[13] | Stomach | 2005 | 1033 | 6.2% | 3.7% | 98.0% |
| Isomoto <i>et al</i> ^[17] | Stomach | 2009 | 589 | 1.8% | 4.5% | 94.9% |
| Chung <i>et al</i> ^[9] | Stomach | 2009 | 1000 | 15.6% | 1.2% | 95.3% |
| Mannen <i>et al</i> ^[26] | Stomach | 2009 | 478 | 8.2% | 3.6% | - |
| Tsuji <i>et al</i> ^[14] | Stomach | 2010 | 398 | 5.8% | - | - |
| Higashiyama <i>et al</i> ^[31] | Stomach | 2011 | 924 | 3.0% | 4.0% | - |
| Okada <i>et al</i> ^[27] | Stomach | 2011 | 647 | 4.3% | - | - |
| Toyokawa <i>et al</i> ^[32] | Stomach | 2012 | 1123 | 5.0% | 2.4% | 93.5% |
| Goto <i>et al</i> ^[18] | Stomach | 2012 | 1814 | 5.5% | - | - |
| Lim <i>et al</i> ^[16] | Stomach | 2012 | 1591 | 5.9% | - | - |
| Koh <i>et al</i> ^[15] | Stomach | 2013 | 1166 | 5.3% | - | 98.5% |

ESD: Endoscopic submucosal dissection.

Table 2 Previous reports of bleeding after esophageal or colorectal endoscopic submucosal dissection

| Ref. | Organ | Year | Case No. | Postoperative bleeding | Perforation | En block resection |
|---------------------------------------|-----------|------|----------|------------------------|-------------|--------------------|
| Ono <i>et al</i> ^[25] | Esophagus | 2009 | 107 | 0.0% | 4.0% | 100.0% |
| Isomoto <i>et al</i> ^[19] | Esophagus | 2013 | 291 | 0.7% | 0.0% | 99.7% |
| Tsujii <i>et al</i> ^[20] | Esophagus | 2015 | 373 | 0.0% | 5.2% | 96.7% |
| Saito <i>et al</i> ^[21] | Colon | 2010 | 1111 | 1.5% | 4.9% | 88.0% |
| Niimi <i>et al</i> ^[79] | Colon | 2010 | 310 | 1.6% | 4.8% | 90.3% |
| Oka <i>et al</i> ^[80] | Colon | 2010 | 688 | 1.7% | 3.3% | - |
| Toyonaga <i>et al</i> ^[22] | Colon | 2012 | 1143 | 1.2% | 1.4% | 99.3% |
| Takeuchi <i>et al</i> ^[81] | Colon | 2012 | 348 | 4.6% | 2.3% | 91.1% |
| Lee <i>et al</i> ^[24] | Colon | 2013 | 1000 | 0.4% | 5.3% | 97.5% |
| Nakajima <i>et al</i> ^[23] | Colon | 2013 | 816 | 2.2% | 2.0% | 94.5% |

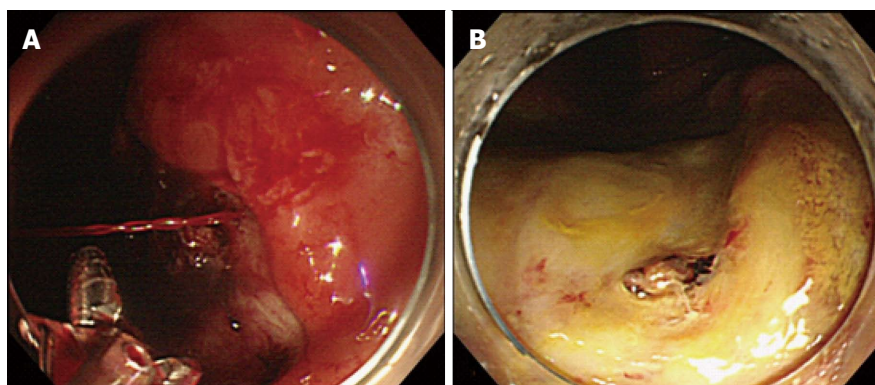


Figure 1 Spurting bleeding from visible vessel on the day after gastric endoscopic submucosal dissection (A) and successful hemostasis by using hemostatic forceps (B).

still insufficient. It is a clinically important, but unsolved question whether antithrombotic agents increase the risk of post-ESD bleeding. Several retrospective studies have shown that antithrombotic agents as a whole are risk factors for post-ESD bleeding^[14,15,33]. Adversely, there is also data which suggests that antithrombotic agents do not significantly increase post-ESD bleeding^[26,27,29,32]. However, the types of antithrombotic agents and cessation periods differed among these studies. Each antithrombotic agent has its own mechanism and carries a different risk of bleeding. So the post-ESD bleeding risk for each agent must be analyzed individually.

Aspirin is known to be one of the most commonly administered antiplatelet agents. Initial reports demonstrated the safety of colonoscopic polypectomy in patients taking aspirin^[39-41]. Similarly the rate of post-ESD bleeding does not significantly increase with the cessation of aspirin from one week before ESD^[16,42]. Although available data concerning continued aspirin use is still lacking, guidelines permit ESD without aspirin cessation in patients with a high-risk of thromboembolism. Recently, Lim *et al*^[16], Matsumura *et al*^[30] and Sanomura *et al*^[43] reported that the continued use of aspirin did not increase the risk of bleeding after gastric ESD. However, Cho *et*

al^[44] reported that continued aspirin use increased the risk of bleeding after gastric ESD; post-ESD bleeding occurred at a rate of 21.1% (4 out of 19 continued aspirin users). It is controversial whether the continued use of aspirin is a risk factor of post-ESD bleeding. More accumulation of data on ESD without aspirin cessation is required.

As for thienopyridine, recent guidelines recommend cessation of thienopyridine from at least 5 d before ESD, according to the data that continued use of thienopyridine increased the rate of bleeding after colon polypectomy^[45]. Although data on thienopyridine monotherapy during the ESD procedure is insufficient, there are two available reports concerning gastric ESD in patients receiving dual antiplatelet therapy. Thienopyridine is principally used in dual antiplatelet therapy (DAPT) in patients undergoing implantation of drug-eluting coronary stents. Tounou *et al*^[46] reported that DAPT markedly increased the rate of post-ESD bleeding (35.5%). Ono *et al*^[47] reported a prospective study regarding bleeding after endoscopic procedure including polypectomy, EMR and ESD. Twenty-eight patients continuing aspirin therapy during endoscopic treatment were enrolled in this study, and 7 patients experienced major bleeding events after ESD (stomach: 6, colon: 1). Subanalysis of gastric ESD showed that all the 6 cases of post-ESD bleeding occurred after the resumption of thienopyridine derivatives^[47]. These reports showed that DAPT is a significant risk factor of bleeding after ESD.

Heparin bridge therapy is commonly performed during cessation of warfarin in patients with a high risk of thromboembolism, but evidence concerning whether heparin bridge therapy can prevent thromboembolism is lacking, and in addition heparin bridge therapy is known to increase the incidence of bleeding events^[48]. Two retrospective studies showed that post-ESD bleeding in patients undergoing heparin bridge therapy occurred at the rate of 23% to 38%^[30,49]. Additionally, Douketis *et al*^[50] reported a randomized trial to evaluate the risks of thromboembolism and bleeding events after operations or other invasive procedures in patients taking warfarin for chronic atrial fibrillation or flutter. This study suggested that forgoing bridging anticoagulation is non-inferior to perioperative heparin bridging for the prevention of arterial thromboembolism and decreases the risk of major bleeding^[50]. Therefore it may be necessary to reconsider whether bridging is necessary for the management of patients taking warfarin.

Recently direct oral anticoagulant drugs (DOACs) have become increasingly used in clinical practice, and the association between DOACs and the risk of post-ESD bleeding needs to be assessed. However, data about DOACs is still being accumulated and is currently still insufficient. The risk of DOACs for post-ESD bleeding remains to be investigated hereafter.

Resection size and other factors

There have been several reports that specimen size > 40 mm is a significant risk factor for post-ESD bleeding^[9,15,27,30]. Owing to the acceptance of expanded indications for larger lesions, there have been increasingly more cases of large ESD in our practices^[51-53]. The reason why larger resection causes more bleeding is simply considered to derive from the fact that more vessels would be exposed on the ulcer bases after large ESD.

Patients receiving hemodialysis are known to be prone to bleed from gastroduodenal ulcers^[54]. A few studies showed hemodialysis is a risk factor for post-ESD bleeding^[30,31,55,56]. Numata *et al*^[55] reported that two ESD-related deaths occurred among hemodialysis patients in an evaluation of ESD outcomes in 63 patients with chronic kidney disease; post-ESD bleeding triggered femoral infarction in one case, and alveolar hemorrhage occurred in the other case. More careful management after ESD may be required for patients on hemodialysis because post-ESD bleeding may lead to secondary adverse events.

Two studies have also shown that long procedure time is an independent risk factor for post-ESD bleeding^[31,32]. A longer procedure time was required in these studies when intraoperative bleeding was frequent and difficult to control, which might mean more vessels exist in the submucosal layer in these cases.

As for the location, it has been generally reported that the lower part of the stomach is a risk factor for post-ESD bleeding. Tsuji *et al*^[14] and Miyahara *et al*^[29] reported that post-ESD bleeding occurred more frequently in the lower part of the stomach than in the upper or middle part. That may be partly because more careful endoscopic hemostasis is required during the ESD procedure in the upper and middle part of the stomach where intraoperative bleeding frequently occurs, which may ultimately prevent post-ESD bleeding^[13,14,29]. Although intraoperative bleeding may be associated to submucosal artery diameters, arteries of the upper and middle part of the stomach are known to be thicker in diameter than in the lower part as evaluated in human resected gastric specimens and dog models^[57,58]. In addition, antral active peristalsis and bile reflux may contribute to a high incidence of post-ESD bleeding in the lower part of stomach^[12,14]. Adversely, Chung *et al*^[9] reported that the upper part of the stomach was a risk factor. They performed hemostasis on all vessels likely to bleed regardless of the location^[9]. Tsuji *et al*^[14] showed that post-ESD bleeding occurred more often when beginners performed coagulation of the ulcer floor after ESD. These discrepancies might occur due to the amount of remnant exposed vessels on the mucosal defect of ESD.

In summary, according to available evidence,

DAPT and heparin bridge therapy significantly increase post-ESD bleeding, but it is unclear whether other antithrombotic agents are risk factors. In terms of other risk factors for post-ESD bleeding, large resection size would be a reliable risk factor, but there have been an insufficient number of prospective studies and there is not enough well-established data. Large-scale prospective analyses concerning this issue are essential.

PREVENTIVE METHODS

Massive post-ESD bleeding occasionally leads to a severe condition that requires blood transfusion, such as hemorrhagic shock^[12]. Therefore, prevention of post-ESD bleeding is imperative. According to previous studies, there are only two well-established effective methods of prevention with supportive evidence: the use of acid secretion inhibitors and preventive coagulation of the ESD-induced ulcer bed.

Acid secretion inhibitors

Acid secretion inhibitors including proton pump inhibitors (PPI) or histamine-2 receptor antagonists (H2RA) are normally used to facilitate healing of ulcers after gastric ESD. It is still unclear whether PPIs can reduce post-ESD bleeding more effectively than H2RAs although several studies have reported that PPIs may be superior to H2RAs^[59-63].

Niimi *et al.*^[64] reported that 2-wk administration of PPI resulted in 80% of the transitional rate to scarring-stage ulcers at 8 wk after ESD. The study suggested 2-wk administration of a maintenance dosage of PPI may be sufficient in cases without deteriorating factors such as concomitant use of antithrombotic agents or ulcerative findings in the tumor. Further studies are required to determine optimum doses and duration of PPI administration.

Preventive hemostasis

Endoscopic preventive coagulation or clipping after ESD may prevent post-ESD bleeding. Takizawa *et al.*^[12] reported that post-ESD coagulation of visible vessels (PEC) prevented post-ESD bleeding (with PEC, 3.1% vs without, 7.1%, $P < 0.01$). Mukai *et al.*^[65] reported that PEC plus artery-selective clipping may reduce delayed bleeding after gastric ESD (PEC, 4.5% vs PEC plus artery-selective clipping, 1.3%, $P = 0.17$). Uedo *et al.*^[66] reported that Doppler US may be helpful to search vessels in the post-ESD ulcers.

However, repeated coagulation by hemostatic forceps can lead to coagulation syndrome or delayed perforation^[67]. A patient with coagulation syndrome presents fever, abdominal pain or leukocytosis as a result of electrocoagulation injury to the gastrointestinal wall. Therefore, endoscopists should take care not to perform excessive coagulation.

Second-look endoscopy

It was originally reported that a second-look endoscopy (SLE) after the initial endoscopic hemostasis for peptic ulcer bleeding significantly reduces the risk of recurrent bleeding^[68]. According to such findings, SLE after ESD is performed in many facilities in Japan. However, recent studies have implied that SLE has little influence on the prevention of post-ESD bleeding^[28,69]. Mochizuki *et al.*^[70] reported that SLE was not routinely recommended for patients with an average bleeding risk (the incidence of postoperative bleeding of SLE group vs non-SLE groups; 5.4% vs 3.8%). On the other hand, Jung *et al.*^[71] reported the efficacy of SLE with prophylactic hemostasis.

Nishizawa *et al.*^[72] systematically evaluated the efficacy of second-look endoscopy for gastric ESD, and they concluded in their systematic review and meta-analysis that second-look endoscopy has no advantage for the prevention of post-ESD bleeding in patients without a high risk of bleeding.

As for patients at low-risk for post-ESD bleeding, it seems that SLE is not routinely recommended. However, there is insufficient data to evaluate the efficacy of SLE in patients with a high risk of post-ESD bleeding.

Even with the above mentioned preventive methods, the rate of postoperative bleeding is still approximately 4.5%^[4]. Therefore, the development of a novel technique that decreases post-ESD bleeding more effectively is essential.

NEW METHODS

In order to prevent post-ESD bleeding, methods of closing or shielding the ESD-induced ulcer seem to be promising. As for the closing method, conventional clipping closure is technically difficult in cases where the mucosal defect is large. Lee *et al.*^[73] reported that mucosal closure with a detachable snare and clips supports earlier healing of ulcers after ESD. Kantsevov *et al.*^[74] reported that endoscopic suturing closure is a feasible technique which can eliminate the need for hospitalization after the ESD procedure.

Recently, the utility of a shielding method using polyglycolic acid (PGA) sheets and fibrin glue to manage ulcers after ESD procedure has been reported. PGA sheets are widely used in the surgical field as an absorbable material to reinforce suturing. Takimoto *et al.*^[75] originally reported the efficacy of shielding a mucosal defect after duodenal ESD using PGA sheets and fibrin glue to prevent delayed perforation. Furthermore, Tsuji *et al.*^[76,77] also reported the possibility of reducing postoperative adverse events, such as post-ESD bleeding or delayed perforation (Figure 2). In addition to PGA shielding, other shielding methods have been reported. There has been a report concerning bio-sheet graft therapy for post-ESD ulcer

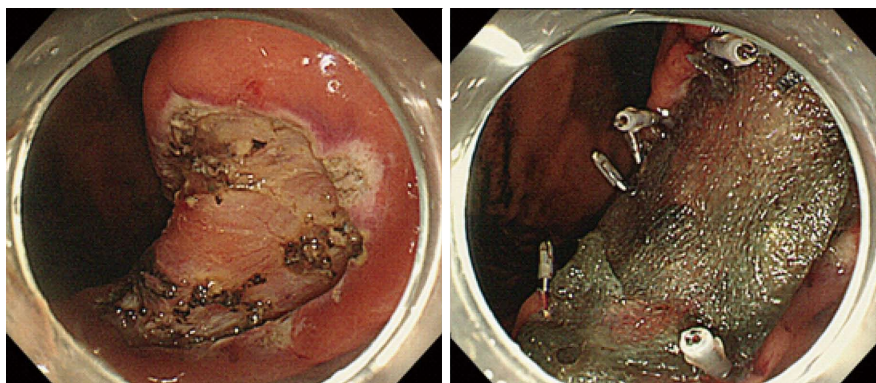


Figure 2 Polyglycolic acid sheets with fibrin glue was applied to the artificial ulcer after gastric endoscopic submucosal dissection by clip and pull method^[82].

in an animal experiment^[78]. According to the study, this bio-sheet graft therapy might be effective in attenuating the degree of inflammation in the ESD-induced ulcers.

However, there has been no randomized controlled trial to investigate the efficacy of these novel methods to prevent postoperative bleeding. Therefore, further research on its efficacy is required.

CONCLUSION

Although ESD has been established as an excellent method of treatment for superficial gastrointestinal neoplasms, the prevention and management of post-ESD adverse events is an issue still to be solved. Especially, controlling bleeding after ESD should be considered one of the top priorities because its occurrence rate is relatively high and sometimes leads to a severe condition. It is imperative for all endoscopists who perform ESD to get acquainted with the risk factors of post-ESD bleeding. To date, some risk factors, such as antithrombotic drug use and large resection size, have been recognized, but optimum management of these risk factors is still to be clarified. Concerning prevention of post-ESD bleeding, PEC and PPI use are widely established as effective preventive methods, but have not been able to prevent bleeding completely. Currently there are several ongoing studies concerning novel techniques for preventing bleeding with the ultimate goal of achieving zero risk for post-ESD bleeding. Further research is required.

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2016 Liver Transplantation: Global view

Predictive factors of short term outcome after liver transplantation: A review

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Abstract

Liver transplantation represents a fundamental therapeutic solution to end-stage liver disease. The need for liver allografts has extended the set of criteria for organ acceptability, increasing the risk of adverse outcomes. Little is known about the early postoperative parameters that can be used as valid predictive indices for early graft function, retransplantation or surgical reintervention, secondary complications, long intensive care unit stay or death. In this review, we present state-of-the-art knowledge regarding the early post-transplantation tests and scores that can be applied during the first postoperative week to predict liver allograft function and patient outcome, thereby guiding the therapeutic and surgical decisions of the medical staff. Post-transplant clinical and biochemical assessment of patients through laboratory tests (platelet count, transaminase and bilirubin levels, INR, factor V, lactates, and Insulin Growth Factor 1) and scores (model for end-stage liver disease, acute physiology and chronic health evaluation, sequential organ failure assessment and model of early allograft function) have been reported to have good performance, but they only allow late evaluation of patient status and graft function, requiring days to be quantified. The indocyanine green plasma disappearance rate has long been used as a liver function assessment technique and has produced interesting, although not univocal, results when performed between the 1st and the 5th day after transplantation. The liver maximal function capacity test is a promising method of metabolic liver activity

assessment, but its use is limited by economic cost and extrahepatic factors. To date, a consensual definition of early allograft dysfunction and the integration and validation of the above-mentioned techniques, through the development of numerically consistent multicentric prospective randomised trials, are necessary. The medical and surgical management of transplanted patients could be greatly improved by using clinically reliable tools to predict early graft function.

Key words: Liver transplant; Liver failure; Early allograft dysfunction; Primary non-function; Initial poor function; Outcome predictors; Post operative; Scoring system; Indocyanine green; Liver maximal functional capacity

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Core tip: The shortage of available livers and long waiting lists have led to increased transplantation of marginal organs. The model for end-stage liver disease allocation system distributes transplants to sicker patients, potentially impairing the final outcome. A serious pitfall is the lack of early postoperative tools to predict short-term outcome for grafts and patients after liver transplant. Here, we review the currently available functional tests and clinical scores that assess graft and patient status during the first week after liver transplantation to quickly guide the early postoperative surgical and intensive care management.

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INTRODUCTION

Liver transplant (LT) is a life-saving treatment for several end-stage liver diseases^[1,2]. Access to LT is now generally performed using the Model for End-Stage Liver Disease (MELD) score. Although created for different purposes, MELD is a simple and highly predictive system for 3-mo mortality for patients on the LT waiting list^[3].

Because of the reduced number of available organs, extended-criteria donors (ECD) are now routinely used for recipients with higher MELD scores, who cannot further delay intervention^[4,5], increasing postoperative mortality and complications in some reports^[6,7]. Due to the complexity of the surgical intervention and the critical status of transplanted patients, a large proportion of the overall complications occur within the first postoperative week after LT.

In this review, we will refer to early allograft dysfunction (EAD) as the sum of initial poor function

(IPF) and primary nonfunction (PNF). EAD, sepsis, secondary complications leading to revision surgery (*i.e.*, arterial or venous thrombosis) and the increased morbidity and mortality of transplanted patients prolong intensive care unit length of stay (ICU-LOS) and hospital length of stay (H-LOS), profoundly impacting the cost of patient management^[8-10]. The initial function of the allograft after LT is determined by donor, surgical and recipient factors, causing IPF incidence ranges between 8.7% to 24.7% and PNF incidence ranges between 0.9% to 7.2% in different LT casuistries^[11,12].

Early EAD diagnosis could allow health care professionals to promptly individuate and treat those patients facing the most worrying conditions. To date, we lack efficient techniques to detect the initial signs of EAD during the first few postoperative days (POD). In fact, despite several years of study on this topic, no concordant definition of EAD and PNF can be found in scientific literature, increasing confusion and contrasting results (see Table 1).

Here, we aim to review the state-of-the-art technologies and tests to assess general patient status, initial graft function and risk of PNF and death after LT, with a specific focus on the tools applicable during the first postoperative week.

PRE- AND INTRA-OPERATIVE PARAMETERS

Several scoring systems have been applied pre-operatively to predict LT outcome. Not being the main focus of this review, we briefly describe the most used ones.

MELD uses three objective laboratory parameters (INR, creatinine, and bilirubin). Low discriminatory power^[13,14] relegated it to be just one of the possible factors predicting patient survival rates, along with other sometimes better performing scores^[15]. MELD evolutions, such as MELD-Na^[16,17], D-MELD^[18] and others^[19], did not reach acceptable performances. The analysis of donor characteristics is also fundamental to optimise graft-recipient matching and to predict LT outcome. So, donor-risk index (DRI)^[20] and extended criteria donor score (ECDS)^[21] were proposed. ECDS, DRI and D-MELD, despite providing statistically significant results, had insufficient discriminatory power for short-term graft and patient survival^[22].

Survival outcome following liver transplantation (SOFT)^[23,24] and balance of risk (BAR)^[25] scores were designed to integrate donor, surgical and recipient risk factors (18 and 6 independent variables, respectively). BAR is a simpler score with extremely high specificity (98%) for identifying patients with high mortality risk. The Charlson Comorbidity Index (CCI)^[26] modified for specific LT needs (CCI-OLT) comprehensively assesses recipient clinical status before LT. All of them were affected by low discriminatory power, limiting their

Table 1 Recent definitions of initial poor function and primary non function

| Ref. | IPF | PNF |
|---|---|--|
| Broering <i>et al</i> ^[107] | ALT or AST or GDH > 2000 IU/L FFP substituted for > 5 d postoperatively | Not-life sustaining graft leading to retransplantation or death within POD10 |
| Nanashima <i>et al</i> ^[108] | Two consecutive measurements within POD3: ALT or AST > 1500 IU/L | IPF-induced retransplantation or death |
| Heise <i>et al</i> ^[109] | Scoring system based on ALT, AST, bile output, Prothrombin activity on POD1-3-7-14 (Berlin score ranging from 4 to 8) Berlin C (IPF): 7-8 | |
| Tekin <i>et al</i> ^[110] | On POD7: AST > 1500 IU/L and PT > 20 s | Not-life sustaining graft leading to retransplantation or death within POD7 |
| Ben-Ari <i>et al</i> ^[111] | AST or ALT > 2000 IU/L on POD2 INR > 1.6 on POD2-10 Bilirubin > 10 mg/dL on POD2-10 | Not-life sustaining graft leading to retransplantation or death within POD10 |
| Kremers <i>et al</i> ^[112] | | ALT > 2500 IU/L Glucose < 60 mg/dL INR > 2.5 bile flow < 50 mL/d |
| Pokorny <i>et al</i> ^[113] | On POD5: AST > 2500 IU/L or clotting support > 2 d | Not-life sustaining graft leading to retransplantation or death within POD7 |
| Monbaliu <i>et al</i> ^[114] | | Persisting encephalopathy Irreversible metabolic acidosis Profound hypoglycaemia Severe coagulopathy Insufficient bile production Increased AST |
| Cieślak <i>et al</i> ^[115] | Within POD1-7 AST or ALT > 2500 IU/L or Prothrombin index < 50% | |
| Dhillon <i>et al</i> ^[116] | [(AST+ALT)/2] on POD2: < 285 IU/L: good function 285-986 IU/L: average function > 986 IU/L: IPF | IPF-induced retransplantation or death within POD7 |
| Nemes <i>et al</i> ^[117] | On POD5: [Serum bilirubin (μmol/L)]/[Prothrombin (%)] > 1 | |
| Olthoff <i>et al</i> ^[81] | On POD1-7, one within: Bilirubin ≥ 10 mg/dL on POD7 INR ≥ 1.6 on POD7 ALT or AST > 2000 IU/L within POD7 | |
| Stockmann <i>et al</i> ^[59] and Lock <i>et al</i> ^[60] | Two LiMax readouts during the first 24 h: LiMax = 60-120 μg/kg per hour | Two LiMax readouts during the first 24 h: LiMax < 60 μg/kg per hour |
| Máthé <i>et al</i> ^[118] | Two consecutive measurements within POD3: ALT or AST > 1500 IU/L | IPF-induced retransplantation or death |

Table freely extracted from Olthoff *et al*^[81], Chen *et al*^[112] and Pareja *et al*^[106]. The mentioned studies are cited in chronological order. ALT: Alanine - aminotransferase; AST: Aspartate - aminotransferase; FFP: Fresh free plasma; GDH: Glutamate dehydrogenase; INR: International normalised ratio; LiMax: Liver maximal function Capacity; POD: Postoperative day; PT: Prothrombin time.

usefulness in individual cases, and scarce prediction of very short-term (1 mo) post-LT survival^[27,28].

Several of these studies focused only on patient outcome. For this reason, early retransplantation was a frequent criterion of exclusion in these publications, which consequently do not provide information about graft function and survival.

Intraoperative anaesthetic management and surgical techniques can strongly influence postoperative patient and graft function. Duration of the intervention, difficult arterial anastomosis, high blood loss and red blood cell transfusion^[29], intraoperative hemodynamic instability^[30], cold and warm organ ischemia time^[11,31], ischaemia/reperfusion (I/R) injury^[32] and the need for and number of necessary revision surgeries can have high impacts on graft functional restoration and patient outcome^[10,12].

To assess all of these variables, functional and analytic tests have been applied to LT.

A preoperative score implementing MELD + ICG

improved survival prediction power in patients with intermediate MELD (10-30), who are often more difficult to correctly prioritise for LT^[33]. Moreover, donor indocyanine green plasma disappearance rate (ICG-PDR) before liver removal was the only factor predicting 7-d graft survival (DRI and donor age were not correlated to graft survival)^[34]. Intraoperative ICG-PDR of 10.8%/min measured 60 min after organ reperfusion had the best specificity and sensitivity in predicting the development of severe EAD with an area under receiver operating characteristics (AUROC) = 0.944, performing better than any other clinical and laboratory parameter and with a negative predictive value (NPV) of 99.2%^[35].

Post-reperfusion lactate levels measured intraoperatively showed significant correlation with 3-mo patient mortality. Patients whose lactate values showed no reduction for 2 h after graft vascularisation experienced higher bilirubin levels from POD5 to POD23. These data came from a small study of 15

living donor liver transplant (LDLT) recipients^[36].

POSTOPERATIVE PARAMETERS

These parameters are the main focus of our review. Table 2 summarises the relevant studies mentioned below and their statistics. They are divided in functional tests (ICG-PDR, LiMAX, others), analytic tests [platelet counts, factor V, transaminases, bilirubin, INR, lactates and insulin growth factor 1 (IGF1)] and clinical scores [MELD, acute physiology and chronic health evaluation (APACHE), chronic liver failure - sequential organ failure assessment (CLIF-SOFA) and model for early allograft function scoring (MEAF)].

Functional tests directly quantify hepatic function. It means that they do not only account for patient and donor risk factors, but also estimate graft conservation, intra-operative organ insult and early postoperative graft function, offering a quantitative and comprehensive value of liver activity.

INDOCYANINE GREEN - PLASMA DISAPPEARANCE RATE

ICG has been used for 25 years to estimate liver function^[37]. ICG is a non-toxic dye that can be administered intravenously and detected by transcutaneous non-invasive densitometry^[38,39]. Normal Indocyanine green - plasma disappearance rate (ICG-PDR) values range from 18% to 25%-30%/min^[38,40]. Being water-soluble, its distribution volume equals plasma volume. ICG is extracted by the liver and excreted through the biliary system without undergoing metabolism or recirculation. For this reason, elimination rates are assumed to depend only on hepatic arterial blood flow and liver functionality. Very few allergic/anaphylactic reactions or thyrotoxicosis due to the iodine component of the solution have been reported. For this reason, ICG is considered a safe bedside tool for the dynamic assessment of implanted liver functionality^[41].

During LT intervention, ICG-PDR falls due to anaesthetic drugs causing haemodynamic hypotension and reduced/absent hepatic function (anhepatic phase). Immediately after graft reperfusion, supra-normal ICG-PDR is observed^[39]. Daily quantification of ICG-PDR from immediately after ICU admittance until POD7 has shown a rapid recuperation in values when appropriate graft function recovery was observed^[42,43]. Harmful conditions for the transplanted patient (EAD, hepatic artery thrombosis, acute rejection or sepsis) and mortality have been associated in those with smoother or absent amelioration of test values. The POD on which this difference becomes significant changes from POD1 to POD4 depending on the study. The critical PDR cut-offs found by Receiver Operating Characteristics analysis (ROC) were 9.6%-12.85%/min^[35,42-44].

A score was developed that considered the only

two independent variables correlating with 1-mo mortality or retransplantation within POD7 (the two primary end-points of the study), assigning 1 point for $\text{INR} \geq 2.2$ and 2 points for $\text{ICG-PDR} < 10\%$. When calculated on POD1, it had strong sensitivity (95%) and NPV (94%) for patients scoring 3. These results were confirmed in a validation cohort^[44]. Confirming the utility of integrating common clinical data with ICG-PDR to increase their specificity, a post hoc study showed that preoperative MELD > 25 and $\text{ICG-PDR} < 20\%$ within 6 h after ICU admission provided extremely rapid and sensitive results (up to 100%, AUROC = 0.79) for ICU- and H-LOS and H-mortality^[45].

A prospective study designed specifically for LDLT investigated 30 patients. EAD patients were characterised by a longer ICU-LOS and higher death rate (50%). Both EAD and non-EAD patients faced a decrease in PDR during the first 48 h after LDLT. Already from POD1 up to POD28, the non-EAD group had significantly higher PDR, while the EAD group showed a progressive deterioration of PDR (confirmed by histopathological analysis of the graft parenchyma). Independently by the absolute values, individual trends might be indicative of graft function and clinical complications. No other laboratory data correlated with the EAD diagnosis at any moment perioperatively^[46].

Finally, ICG-PDR was also proposed as a predictive tool for hepatic artery thrombosis and its management^[47].

A primary limitation to ICG-PDR reliability is given by hemodynamic instability (a frequent perioperative condition in LT) and altered hepatic blood flow^[48]. PDR is also altered by clinical conditions that burden the delicate function of an implanted liver, including cholestasis, hyperbilirubinaemia and capillary leakage^[49,50] because ICG and bilirubin use the same plasmatic transporter^[51]. The multiple confounders affecting PDR might explain the poor specificity and positive predictive value detected with this technique. Finally, ICG-PDR reference values vary among different studies (from 9.6% to 20%) and appear to be context-dependent, depending on the POD of evaluation and clinical complications affecting the patients. For example, sepsis is main cause of patient mortality after LT that consistently alters PDR values, increasing the confusion about reliable cut-off values for this technique^[42,52]. As criticised by Stockmann *et al*^[53], the scoring systems chosen to define IPF and PNF in some of these studies^[42-44] may have created biases or result overestimation. Because of the inability to uniformly diagnose these clinical conditions from the actual scores^[54], a better assessment would focus on patient outcome, as performed by Olmedilla *et al*^[44].

LIVER MAXIMAL FUNCTION CAPACITY

Liver Maximal Function Capacity (LiMax) is a real time breath test: ^{13}C methacetin is administered intravenously, selectively metabolised by cytochrome

Table 2 Overview of the studies applying the mentioned techniques specifically about liver transplant

| Technique | Study | Type (P/R) | Primary end-point: | Sample | POD | Cut-off value | AUROC (95%CI) | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|-----------------------------------|---|------------|--|---|-------------------------------|--|--|------------------------------|----------------------------|----------------------------|------------------------------|
| ICG-PDR | Olmedilla <i>et al</i> ^[33] | P | EAD prediction | 172 LT: 31.9% HCC, 29.6% viral, 23.8% alcoholic | 1 | 10%/min | 0.967 (0.915-0.991) | 100 (69-100) | 90.4 (84.7-94.6) | 40.00 | 100.00 |
| | Levesque <i>et al</i> ^[42] | P | EAD prediction | 72 LT (including LDLT) | 0-5 | 12.85%/min | | 90.0 | 97.0 | | |
| | Schneider <i>et al</i> ^[43] | P | Graft loss or patient death on POD30 | 86 LT: 36% viral, 29% alcoholic | 7 | 12.3%/min | 0.729 (0.608-0.850) | 69.0 | 67.0 | 57.00 | 77.00 |
| Preoperative MELD + postoperative | Klinzing <i>et al</i> ^[45] | P | ICU-LOS, mortality | 50 LT | 0 (< 6 h after ICU admission) | MELD > 25, ICG-PDR < 20%/min | 0.79 | 100.0 | 59.0 | | |
| ICG-PDR | | | | | | | | | | | |
| ICG-PDR + INR | Olmedilla <i>et al</i> ^[44] | P | 1-mo mortality or need for retransplantation within POD7 | 332 LT (+77 validations) | 1 | ICG-PDR < 10%/min, INR > 2.2 | 0.76 (0.66-0.86) | 48 (31-66) | 95 (91-97) | 50 (32-68) | 94 (91-96) |
| | | | | | | | | | | | |
| LiMax | Lock <i>et al</i> ^[38] | P | EAD requiring reintervention before POD2 or causing death/retransplantation within POD14 | 99 LT: 32% alcoholic, 23% HCV | 0 1 | 64 µg/kg per hour 43 µg/kg per hour | 0.960 (0.921-0.998) 0.992 (0.975-1.000) | 100 (60-100) 100 (31-100) | 92 (84-97) 100 (94-100) | 53 (27-78) 100 (31-100) | 100 (95-100) 100 (94-100) |
| Platelets count | Lesurtel <i>et al</i> ^[72] | R | Severe complications or 3-mo mortality | 257 LT: 38% HCV | 5 | 60 × 10 ⁹ /L | | 58.0 | 61.0 | | |
| Factor V | Li <i>et al</i> ^[70] | R | EAD prediction | 234 LDLT: 45% HCC | 2 | 68 × 10 ⁹ /L | 0.678 | 73.0 | 59.0 | | |
| | Zulian <i>et al</i> ^[76] | R | Graft failure within POD90 | 105 LT: 79.5% HCC, 76.2% HCV | 2 | 41.50% | 0.650 | 42.9 | 87.9 | 35.30 | 90.90 |
| AST | Robertson <i>et al</i> ^[78] | P | Graft loss at POD90 | 1091 LT: 22% HCV | 3 | 2 cut-offs: 106.5 IU and 2744.5 IU | 0.739 (0.663-0.814) | | | 34.62 | 99.45 |
| Bilirubin | Wagener <i>et al</i> ^[80] | R | Graft loss or death within POD90 | 572 LT: 51.9% HCV | 2 | 6.55 mg/dL | 0.809 (0.742-0.877) | 72.5 | 70.4 | | |
| Bilirubin, INR and transaminases | Olthoff <i>et al</i> ^[81] | R | EAD definition to predict mortality and graft loss | 300 LT | 7 | Bilirubin > 10 mg/dL, INR > 1.6, ALT or AST > 2000 IU/mL | 0.75-0.78 | | | | |
| Lactates | Wu <i>et al</i> ^[84] | P | EAD prediction | 222 LT: 50% HBV, 41% HCC | 1 | 24.80% | 0.961 (0.948-0.974) | 95.5 | 88.9 | | |
| IGF-1 | Bassanello <i>et al</i> ^[88] | P | Explore GH/IGF-1 axis changes during the perioperative course of LT | 15 LT: 52% viral, 20% alcoholic | 7 | n.a | | | | | |
| | Salso <i>et al</i> ^[90] | R | 90-d patient survival | 30 LT: 40% HCV, 20% HBV | 15 | 90 mUI/mL | 0.920 | 86.0 | 87.0 | | |
| | Nicolini <i>et al</i> ^[89] | P | 3-yr actual survival | 31 LT: 42.5% HCV | 15 | Normal values classified according to Immunolite 2000® system reference-ranges | | | | | |

| | | | | | | | | | |
|--------------|--------------------------------------|---|--|--|-------------------|--------|---------------------|-------|------|
| MELD | Wagner <i>et al</i> ^[80] | R | Graft loss or mortality within POD90 | 572 LT; 51.9% HCV | 5 | ≥ 19 | 0.812 (0.739-0.886) | | |
| | Toshima <i>et al</i> ^[91] | R | Graft loss or mortality within POD180 | 217 LDLT; 47.9% HCV | 2 | ≥ 19 | 0.779 | 68.2 | 79.5 |
| | | | | | 7 | | 0.933 | 100.0 | 31.0 |
| MELD lactate | Cardoso <i>et al</i> ^[92] | P | Mortality within POD30 | 58 LT; 43% HCV, 26% alcoholic | 1 h after surgery | 26.3 | 0.800 | | |
| APACHE IV | Hu <i>et al</i> ^[109] | R | Mortality | 195 LT | 1 | ≥ 55.5 | 0.937 (0.892-0.981) | 85.2 | 91.1 |
| SOFA | Wong <i>et al</i> ^[120] | R | 3-mo mortality | 149 LT; 53% HBV | 7 | ≥ 8 | 0.953 (0.902-1.000) | 95.0 | 91.0 |
| CLIF-SOFA | Pan <i>et al</i> ^[104] | R | 1-yr mortality | 323 LT; 62% HBV, 27% hepatoma, 26% HCV | 3 | > 8 | 0.808 (0.729-0.888) | 67.0 | 87.0 |
| | | | | | 7 | | 0.877 (0.813-0.941) | 64.0 | 95.0 |
| MEAF | Pareja <i>et al</i> ^[106] | R | EAD definition to predict 3-mo mortality | 874 LT (+200 validation) | 3 | > 8 | | | |

P450 1A2 (CYP1A2), an enzyme exclusively expressed by hepatocytes, and excreted by ventilation. Quantification of the $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratio after at least 6 h of fasting provides a specific estimation of enzyme kinetics. The systemic liberation of paracetamol is a reaction product. Healthy controls showed normal LiMax values > 315 ng/kg per hour^[55].

LiMax test was first tested in hepatic surgery. The extremely encouraging results^[55] allowed the development of single-patient decision algorithms^[56]. Non-critical but infra-normal LiMax test levels correlated with post-surgical complications, highlighting their relevance in patient monitoring during the postoperative ICU stay^[57]. From these initial studies, the field of investigation moved towards LT.

Ninety-nine LT patients were studied, and non-EAD patients showed significantly higher LiMax within 6 h after LT than EAD patients. The best discriminating cut-off point was 64 $\mu\text{g/kg}$ per hour. False positive patients with late LiMax recovery on POD2-3 showed increased ICU-LOS and hemodialysis, justifying special attention from ICU doctors. No other variables were independently associated with EAD so early (not even ICG-PDR)^[58].

A quantitative and precise definition of EAD was determined using LiMax. Two cut-offs were arbitrarily decided at 60 and 120 $\mu\text{g/kg}$ per hour. PNF was defined as LiMax $< 60 \mu\text{g/kg}$ per hour, IPF was LiMax 60–120 $\mu\text{g/kg}$ per hour, and immediate function was LiMax $> 120 \mu\text{g/kg}$ per hour. Values were measured within 24 h after LIT in the same previous 99 recipients, and post hoc analysis was performed. Using these cut-offs, IPF correlated with biochemical laboratory values (transaminases, bilirubin, INR, creatinine) and higher rates of post-transplant complications (hemodialysis and catecholamine support) but not with H-LOS and 2-year patient and graft survival. Slower restoration of LiMax values was detected in IPF patients up to POD28, while all immediate function patients recovered normal LiMax values by POD5. Three cases of PNF underwent immediate retransplantation. EAD patients were characterised by significantly higher DRI and donor age (no preoperative MELD differences were evaluated), showing postoperative LiMax correlating with both donor characteristics and recipient clinical progression^[59].

Immunosuppression is a principal treatment after LT. Tacrolimus, one of the most frequently used immunosuppressants, is metabolised by hepatocytes. Normal blood concentrations of tacrolimus might be toxic for patients developing EAD. In a following prospective observational study, graft function was quantified by LiMax during the first 5 d after LT. LiMax levels predicted the development of toxic levels of tacrolimus in patients with EAD and tacrolimus under-dosage in those with good graft function^[60]. Immunosuppression modulation of tacrolimus blood concentrations poorly correlates with ICG-PDR^[61].

A major concern about this technique is that it was developed and clinically applied by only a single study group. Although encouraging, few publications are available. The two major publications on LiMax and LT used the same group of 99 LT patients, limiting their significance. Wider, multicentre applications are required to verify reliability in different cohorts and clinical settings. Because of the extreme variability of cytochrome activity depending on external factors, some scepticism may arise, and extensive studies to confirm the inter-individual reliability and standardised cut-off values of this specific technique are needed.

OTHER FUNCTIONAL TESTS

Several other functional techniques were tested decades ago with promising results, such as the lidocaine-monoethylglycinexylidide (MEGX) injection test^[62,63] and the galactose elimination capacity^[64]. Unfortunately, hepatic blood flow, genetically-determined variation of enzymatic function, and different hepatic functionality consequences due to different pathologies, treatments and other external factors (such as drugs, dietary habits, nutritional status and coexisting pathologies) resulted in extremely high inter-individual variability and the impossibility in defining reliable cut-off values. Frequently, non-univocal experimental results and time-consuming techniques discouraged further clinical experimentation, and they never reached bedside utilisation.

Other breath tests based on stable isotopes or on specific mitochondrial functions were proposed to assess liver function capacity, but most of these techniques have never been used in specific correlation with the assessment of liver transplant patients and graft function^[65].

PLATELET COUNTS

Platelets are a blood component with a wide range of acute conditions, including inflammation, infections, tissue insults from I/R injury and tissue regeneration, acting an active role in LT^[66]. LT candidates frequently present low platelet counts due to congested splanchnic circulation, increased mechanical stress and reduced bone marrow activity. These causes are not immediately reverted by LT^[67].

Interestingly, red blood cells, plasma and platelet transfusions have been correlated with negative outcome after LT and might be associated with a lower nadir in postoperative platelet count^[68-70]. Thrombocytopenia after LT is associated with increased EAD, early development of bacterial and fungal infections (before POD14), and patient mortality^[68,71].

In a retrospective study, patients were divided into two groups based on their platelet count on POD5 after LT, and a cut-off value was set at 60×10^9 platelets/L based on the best AUROC. MELD > 25 and platelet count < 60×10^9 platelets/L were the best predictors of severe postoperative complications and mortality, increased ICU-LOS and H-LOS independently of preoperative levels and intraoperative transfusions. POD5 platelet count showed to be a reliable predictor of short-term outcome after LT (within POD90). Unfortunately, platelet counts decrease from immediately post-transplant until POD 3-6, returning to preoperative levels by week 1-2, thus severely limiting the utility and power of this parameter in early graft assessment^[72].

Another retrospective study investigated the role of postoperative platelet count in 234 LDLT patients^[70]. In this specific field of hepatic surgery, platelet

transfusions have been reported to improve graft regeneration^[73,74]. A cut-off of 68×10^9 platelets/L for the immediate postoperative platelet count was determined following ROC analysis. Values lower than this cut off were found to be a risk factor for IPF incidence and severe complications. No differences were found for the 90-d mortality rate, PNF and ICU-LOS.

Weak statistics in few retrospective studies limit our knowledge of the meaning of platelet count post-LT. Finally, it is not easy to understand if postoperative thrombocytopenia is a cause or a consequence of EAD.

FACTOR V

Coagulation factors I, II, VII, VIII, IX, X, and XI, protein C, protein S and anti-thrombin are produced by the liver. Thus, coagulation relies heavily on its conserved synthetic capacity. Factor V is a cofactor for the prothrombinase complex, that activates prothrombin to thrombin, interacts with several coagulation factors, and also modulates the anticoagulant pathway by down-regulating factor VIII activity. Factor V does not depend on vitamin K for its production and is characterised by a short half-life (< 24 h), strictly tracing liver function at the moment of its dosage. For this reason, it has been found to be a good prognostic marker of fulminant liver failure^[75].

When specifically tested for LT, factor V measurement on POD2 was retrospectively found to be an independent predictor for both 90-d graft function and overall survival^[76]. A cut-off was set at 41.5% after ROC analysis. No differences in preoperative data distinguished the groups with high vs low POD2 factor V. Plasma transfusions did not differ significantly between these two groups and therefore did not create misleading artefacts in data interpretation. Good specificity (87.9%) and NPV (90.9%) were detected for 3-mo graft survival. Also the 5-year patient survival rate correlated with Factor V levels on POD2.

TRANSAMINASES, BILIRUBIN AND INR

Aspartate and alanine transaminases (AST and ALT) are enzymes involved in amino acid metabolism. ALTs are more liver-specific, but ASTs occur at higher concentrations in the liver^[77]. IPF and PNF definitions in the early 1990s were based on extremely high levels of transaminases as an estimate of hepatic damage and hepatocellular lysis. Then, both bile production/bilirubin levels and prothrombin time (PT) were investigated, focusing attention on the synthetic activity and functional state of the liver.

Recently, a large prospective study found that AST on POD3 plus AST and ALT on POD7 are predictors of early (within POD90) graft failure from both general and liver-specific causes. The best AUROC with an extremely high NPV (99.34%) was detected for AST on POD3. Accordingly, patients were divided into 4

risk groups, which also correlated with 1-, 3- and 12-mo mortality rates, ICU-LOS, renal replacement therapy and incidence of septic complications. For the first time, non arbitrary cut-off levels and POD of measurement for transaminases were defined. Moreover, AST on POD3 mirrors hepatic damage due to long times in performing vascular reconstruction, long cold ischemia times and preoperative MELD values^[78].

After deceased cardiac donor LT, non-anastomotic biliary strictures are a major cause of ALT peak ≥ 1300 IU/L and EAD, likely because of longer ischemia time and immunological causes^[79].

A monocentric retrospective analysis, postoperative AST (POD1-5), creatinine (POD3-7), INR (POD0-7), bilirubin (POD0-7) and MELD scores (POD0-7) strictly correlated with graft dysfunction within POD90. The best AUROCs, for MELD on POD5 and bilirubin on POD2, did not statistically differ in their predictiveness. Thus, total bilirubin > 6.55 mg/dL on POD1-2 should alert clinicians^[80].

Olthoff *et al.*^[81] excluded INR and bilirubin up to POD7, suggesting that those values might still reflect recipient pre-transplant status and not graft functionality. However, AST and ALT were evaluated daily on POD1-7, immediately reflecting eventual graft injury. Their EAD definition showed good prediction of 6-mo mortality, with a relative risk = 10.7 (95%CI: 3.5-31.9). Similar results were found for 6-mo graft loss.

The rapid kinetics of AST and ALT should be considered to assess graft viability and trial endpoints in LT. The majority of peak ASTs are detected at 6 h post-reperfusion, with a time window between 5 and 11 h. These could be missed if AST determination relies only on routinely taken samples, usually after ICU arrival. The precise timing of the first blood sample or a specific time window should be indicated to make the results more reproducible and avoid erroneous classification of serum transaminases^[82].

LACTATES

Lactates, the waste products of cellular metabolism, are mainly metabolised by the liver. Thus, liver function and its restoration after LT might be reflected by abnormally elevated lactate levels. A damaged liver can itself be a source of lactate.

ICU survival has been predicted by calculating the percentage of lactate reduction between the time of admission and 6 h after admission^[83]. An observational prospective study divided 222 consecutive LT patients into two groups, those who developed EAD and those who did not. Initial absolute lactate values did not differ between the two groups, but clearance during the first 6 hours of ICU stay was significantly higher in the non-EAD group. AUROC of 0.961, for a cut-off point of 24.8% of clearance, was much higher than other significant parameters; an odds ratio of 169 (95%CI:

52.49-544.13) was calculated for the prediction of EAD. The group with a lower clearance showed higher in-hospital mortality but no differences in 1-year mortality. So, early measurement of lactates allows immediate functional graft assessment rather than a medium- or long-term clinical outcome prediction. The ease of the technique makes it readily available at the bedside^[84].

IGF1

Hepatic dysfunction affects several biochemical processes taking place in this organ. One fundamental endocrine axis involving the liver, is the Growth Hormone (GH) - IGF1 axis. Liver damage measured through clinical scales such as MELD or Child-Pugh correlates with decreased levels of IGF1, which is synthesised by the hepatocytes, and consequently with increased GH levels^[85,86]. IGF-1 and GH levels correlate with common enzymes to assess liver function and to describe post-LDLT liver regeneration in both donors and recipients^[87]. In LT, peri-operative quantification of IGF-1 showed a dramatic decrease during the anhepatic phase, with levels already significantly rising 30 min after the completion of the surgery and completely normalising between POD7 and 28. From POD7, significantly lower levels were detected in patients who developed IPF^[88]. Consequently, the prospective 3-years follow up a small group of 31 transplanted patients showed that 18 of them already had normalised IGF-1 levels on POD15, and their actual 3-year survival rates were significantly improved. Decreased levels of IGF-1 during the whole 1st year after LT were found in patients transplanted with livers from donors older than 65. From POD90, low IGF-1 levels significantly correlated with increased ECD score^[89]. The IGF-1 serum test is a quick, inexpensive and reproducible immunometric assay, giving this parameter an advantage over other molecules in the prediction of initial graft function. Unlike ICG-PDR, IGF-1 determination is not influenced by patient hemodynamic instability or hyperbilirubinaemia.

Unfortunately, in the above mentioned studies, significant results were found only starting from POD7^[88] and POD15^[89] respectively. Similar results were found in another retrospective study with a small sample of 30 LT patients who only found predictive IGF-1 values for the 90-d survival rate on POD15^[90]. Thus, to date this technique cannot be applied during the first postoperative week in the ICU to guide treatments and strategies.

POSTOPERATIVE MELD SCORE, MELD LACTATE

The poor performance of preoperative MELD in predicting post-LT graft and patient survival has already been

discussed. Despite this consideration, postoperative MELD has been used repeatedly for this purpose because of its simplicity and easily measurable variables. It performs well as a predictor of 90-d graft failure (defined as patient death or re-transplantation). MELD > 18.9 on POD5 had the best performance and better predictive power of all commonly available data. No significant difference was found between MELD values recorded from POD0 to POD7, but the best AUROC (> 0.8) was on POD5^[80].

After LDLT, postoperative MELD < 19 on POD2 (up to POD14) performed better than preoperative MELD in predicting 6-mo graft survival, with a peak of AUROC (0.933) and specificity (100%) on POD7^[91]. The MELD lactate score 1 h after the end of the LT surgery was found to be a predictor of 30-d patient survival. At that time of early patient recovery, lactates are able to account for I/R liver damage, donor liver problems (not always predictable before LT), general recipient status, infections and surgical problems. Most non-survivors with high MELD-lactate scores died in ICU within a few days after LT^[92].

APACHE

This score, created in the 1980s^[93] and successively modified until APACHE IV^[94-96], aimed to predict mortality in critically ill patients. Not being specifically designed for transplanted patients, when used alone APACHE II displays overestimation problems regarding patient mortality and low AUROC values^[97,98]. Then, a specific correction factor for LT was created to correct APACHE (APACHE-LT). When APACHE II-LT was calculated on POD1, it performed better than any other scoring system, with statistically non-significant differences between predicted and observed mortality^[99].

Contrasting results about APACHE II came from different clinical studies, likely because of different sample groups in different geographical regions^[98,100,101]. This confirmed the weakness of clinical scores applied alone in predicting patient outcome.

CLIF-SOFA

SOFA is a scoring system allowing the quantification of the number and severity of apparatus dysfunction in a critically ill patient^[102]. An adjusted SOFA score accounting for end-stage liver disease was defined CLIF-SOFA^[103].

When tested to predict 3- and 12-mo mortality in an unicentric cohort of 149 LT patients retrospectively divided in 1-year survivors and non-survivors, SOFA had the best discriminatory power (higher than MELD). 323 patients from the same cohort were then analysed using CLIF-SOFA. An excellent AUROC was detected, with significantly best discriminatory power on POD1-7 with respect to MELD and SOFA. The best AUROC (0.877) for CLIF-SOFA occurred on POD7. Significantly

different cumulative survival rates for CLIF-SOFA ≤ 8 vs CLIF-SOFA > 8 were detected^[104].

These two studies come from a single group, and more than 50% of patients in both studies had Hepatitis B (and 27% hepatomas in the latter case) that required LT, reflecting the geographical area of the provenance of these studies. These facts might affect the results and reduce their reproducibility.

After LDLT, SOFA score on POD7 had the highest power to predict 3-mo mortality^[105].

MEAF

A biochemical-based scoring system was developed after retrospectively collecting data from a unicentric database (829 recipients) and then tested on a validation group (200 recipients) from a different centre^[106]. Primary end-points were patient mortality at 3, 6, and 12 mo after LT, PNF and EAD. The highest ALT, AST, INR and PT levels within the first 3 postoperative days and bilirubin on POD3 were found to reliably describe EAD. MEAF is calculated through a non-linear regression model and is completely calculable on POD3. Slight evidence of correlation of the MEAF score with 3-mo mortality was found (confidence interval 1.01-1.41). However, the 3-mo mortality rate rose to 40.6% for patients with MEAF > 8. A sharp increase in the development of PNF and EAD was registered for those with MEAF > 7. Significant correlation of MEAF with ICU and hospital stay was also found. All data were confirmed and strengthened in the validation cohort. The nature of MEAF makes it a more flexible tool than those using pre-established cut-offs, likely increasing its value with respect to older EAD definitions.

The simplicity of this model and its rapid applicability on POD3 make it a suitable candidate for predicting PNF, 3-mo graft loss and patient mortality.

CONCLUSION

Although there are a wide variety of laboratory and functional tests available to directly or indirectly quantify graft function after LT, it is still difficult to predict graft and patient survival after this major surgical intervention. Many efforts were made to individuate diagnostic EAD criteria or critical patient conditions early (within the first few PODs), which might be essential for a successful outcome of LT. Rapid and precise instruments to understand initial graft function and other comorbidities affecting the recipient could guide the medical staff to more effective, and aggressive when necessary, strategies to support both liver and general condition after LT. Encouraging results come from functional tests like LiMax test and ICG-PDR which could not only predict the outcome but even indicate the best therapeutic decisions. Although several limitations and contradictions have been illustrated, MEAF and other scoring systems, might

become reliable, simple and cheap predictors during the first PODs.

Unfortunately, few techniques have revealed consistent initial results probably for their retrospective, monocentric nature, for the small number of subjects studied and for the low predictive power. The lack of unique definitions of reference values and occasional high economical costs also limit their usage. By critically considering the statistics and the clinical samples reported in this review, it might be possible to integrate different scoring systems and functional tools, instead of using single indices, to better assess early graft function offering help in surgical and medical early postoperative patient management. The creation of such a complex analysis is beyond the aims of this review and the possibilities of the authors. Multicentric prospective trials should be performed to avoid wasting the resources and clinical knowledge currently available.

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2016 Liver Transplantation: Global view

Selective intestinal decontamination for the prevention of early bacterial infections after liver transplantation

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Abstract

Bacterial infection in the first month after liver transplantation is a frequent complication that poses a serious risk for liver transplant recipients as contributes substantially to increased length of hospitalization and hospital costs being a leading cause of death in this period. Most of these infections are caused by gram-negative bacilli, although gram-positive infections, especially *Enterococcus* sp. constitute an emerging infectious problem. This high rate of early postoperative infections after liver transplant has generated interest in exploring various prophylactic approaches to surmount this problem. One of these approaches is selective intestinal decontamination (SID). SID is a prophylactic strategy that consists of the administration of antimicrobials with limited anaerobic activity in order to reduce the burden of aerobic gram-negative bacteria and/or yeast in the intestinal tract and so prevent infections caused by these organisms. The majority of studies carried out to date have found SID to be effective in the reduction of gram-negative infection, but the effect on overall infection is limited due to a higher number of infection episodes by pathogenic enterococci and coagulase-negative staphylococci. However, difficulties in general extrapolation of the favorable results obtained in specific studies together with the potential risk of selection of multiresistant microorganisms has conditioned controversy about the routinely application of these strategies in liver transplant recipients.

Key words: Selective intestinal decontamination; Liver transplant; Infection; Gram-negative bacterial infection; Gram-positive bacterial infection; Multiresistant

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Core tip: Liver transplantation has become the treatment of choice for many liver diseases. It is currently a routine procedure but is still associated with significant morbidity being infectious complications the leading cause of death. Selective intestinal decontamination (SID) is a prophylactic strategy that consists of the administration of non-absorbable or systemic antibiotics with scarce anaerobic activity in order to prevent or minimize the impact of endogenous infections by potentially pathogenic microorganisms. In this review, we focus on the knowledge regarding the current role of SID in liver transplant recipients. Multiple studies have evaluated the role of SID in the critically ill patient, and several observational studies, randomized clinical trials and a meta-analysis have focused in liver transplantation. Our aim is to consolidate the current literature to better outline the impact of SID in the prevention of infections in this setting.

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INTRODUCTION

Since 1963, when Starzl *et al.*^[1] performed the first successful liver transplantation, the outcomes and long-term survival rates after liver transplantation have significantly improved over the last 5 decades. Major advances in transplantation biology, organ procurement and preservation, techniques of surgical implantation, immunosuppressive therapy and the prevention and management of infection have made liver transplantation the treatment of choice in many liver diseases^[2-5]. Although long-term survival rates have been currently improved^[6], this procedure it is still associated with significant morbidity, being infection one of the most feared complications.

Liver transplant patients are highly vulnerable to bacterial infection, particularly due to gram-negative organisms. It is believed that most of these infections are endogenous and arise from aerobic gram-negative bacteria and yeasts that have previously colonized the oropharynx, stomach, and bowel^[7]. This has led to the development of selective intestinal decontamination.

SID aim to eradicate potential pathogenic microorganisms (PPM) from the digestive tract, especially aerobic gram-negative bacilli (AGNB), but also *Staphylococcus aureus*, *Enterococcus* and yeasts, in order to prevent infections in patients at high risk of infection.

In general, the target of SID is to prevent or eradicate the state of gastrointestinal carrier by PPM keeping other microbial commensal flora as intact as possible since this it is assumed to have an important role

in the resistance to colonization by PPM. The final endpoint of this strategy should be reduction of mortality and morbidity associated with infection in these high-risk patients.

ABNORMAL COLONIZATION OF THE GASTROINTESTINAL FLORA AND OROPHARYNGEAL

After the introduction of antibacterial agents it has been postulated that the usual gastrointestinal and oropharyngeal commensal flora (mainly anaerobic flora) has an important role in regulating the proliferation of flora that includes aerobic PPM. Anaerobic antibiotics secreted inside the colon lumen can exert a suppressive effect on endogenous commensal flora, with consequent overgrowth of *S. aureus*, AGNB and, as recently stated, microorganisms with lower pathogenicity as enterococci, including *E. faecium* and vancomycin-resistant *Enterococcus*^[8,9].

Healthy mammals are able to eliminate very high concentrations of gram-negative bacilli (including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* y *Enterobacter cloacae*) contaminating the water they drink^[10]. The concept of colonization resistance has been defined through experimental animal models as the amount of inoculated bacteria in the colon necessary for converting in carriers of abnormal flora at least 50% of studied animals^[10].

The use of antibacterials disturbing protective commensal flora favors overgrowth of abnormal flora in the gastrointestinal tract^[11]. Although there are differences in the ability of different antibiotics to select potentially PPM, none of the currently available antibiotics are completely safe in this regard^[12]. Antibacterials with higher bactericidal activity against anaerobic flora are more prone to eradicate bacterial flora (*i.e.*, treatment with amoxicillin is associated with higher disruption of colonic flora than cephalosporins). This effect is more relevant with broad spectrum beta-lactams as amoxicillin-clavulanate, piperacillin-tazobactam and ceftriaxone. In contrast, aminoglycosides have minimal effect on the gastrointestinal commensal flora. Despite its low anaerobic activity, norfloxacin, ciprofloxacin and levofloxacin favor the overgrowth of yeast by eliminating aerobic flora and, therefore, decreasing oxygen consumption generating an unfavorable microclimate for the growth of anaerobic flora^[13].

Some underlying diseases have an evident influence on the ease for developing disorders in bacterial flora. Higher rates of oropharyngeal colonization and/or gastrointestinal by AGNB have been reported in patients with diabetes, alcoholism, chronic obstructive pulmonary disease or liver disease^[7].

In patients admitted to intensive care units (ICUs) a high proportion of abnormal colonization by AGNB ranging 30%-50% is observed, depending on the severity of patients^[14-16]. It is assumed that most of

the patients admitted to the ICU develop abnormal colonization during the first week of admission^[17]. Other factors that have been implicated with abnormal resistance to colonization in these patients are: (1) Anatomical integrity of the mucosa; (2) Conservation of pH in saliva and stomach; (3) Conservation of motility through the masticatory act, swallowing and peristalsis; (4) Presence of immunoglobulin A in the mucous membranes; and (5) Conservation of usual commensal flora in the mucous, mainly anaerobic flora.

SELECTIVE INTESTINAL DECONTAMINATION

The first description of the use of antibiotics to eliminate abnormal oropharyngeal and gastrointestinal flora dates back to the early 80's and initially consisted in the enteral administration of non-absorbable antibiotics (polymyxin and tobramycin) which eliminated colonization by AGNB without significantly affecting the normal commensal anaerobic flora, coining the concept of selective intestinal decontamination (SID)^[18]. It was subsequently shown that the addition of amphotericin B or nystatin allowed furthermore better control of the overgrowth of yeast without affecting the ecology of the patient^[12]. Other studies have shown that SID in oropharyngeal and intestine was able to control migration and translocation of the PPM at the lower respiratory tract and even in blood^[19,20]. This effect has been demonstrated particularly beneficial in critically ill patients as they present dysfunction of all the mechanisms of defense against abnormal colonization of the mucous membranes. Topical antibiotics against gram positive have also been used, mainly against oxacillin-resistant *S. aureus* in paste or gel formulations^[21].

Two forms of decontamination are currently globally distinguished: (1) Selective oropharyngeal decontamination (SOD) with non-absorbable topical antibiotics as a paste, gel or soluble tablets in the oropharynx^[22,23]. With the application of topical antibiotics in the oropharynx adequate eradication is achieved in about three days. Mouthwashes or oropharyngeal spray applications appear unsuitable due to insufficient contact time of the antibiotic with colonized mucosa; and (2) Gastro-intestinal selective decontamination (SID) either with topical antibiotics in suspension formulations^[24,25] or administration of systemic antibiotics. Compared with oropharyngeal application, the time required for topical antibiotics in the intestine to achieve the eradication effect is more variable since it depends on patient peristalsis, being generally longer (about 7 d)^[26]. The most widely used systemic antibiotics for SID are short courses of 3-4 d of broad-spectrum antibiotics (mainly third generation cephalosporins) or prolonged administration antibiotics with little anaerobicidal activity such as quinolones^[27,28].

SELECTIVE INTESTINAL DECONTAMINATION IN THE LIVER TRANSPLANTATION

In liver transplantation, one of the main complications is bacterial infection, especially by gram-negative organisms. Their frequency varies between 20% and 80%. They contribute substantially to increase hospital stay, as well as hospital costs and are the leading cause of death in this population^[29-31]. Most of these infections occur in the first month after transplantation^[32] and especially during their stay in the ICU^[33]. As mentioned before, it is believed that most of these infections come from the gastrointestinal tract colonization by bacteria and fungi^[7].

Multiple studies have evaluated the role of selective intestinal decontamination in the critically ill patient, including more than 40 prospective randomized trials, with clinical benefits summarized in several meta-analyses^[24,34-40]. This intervention in intensive care units has repeatedly shown reductions in hospital-acquired infection rates (mostly in ventilator-associated pneumonia), and even reductions in overall mortality in some of these studies^[24,36-42]. However, SID remains controversial because of uncertainty regarding its net benefit and concerns about the potential promotion of the emergence of antimicrobial resistance. In a recent meta-analysis no evidence for increased colonization or infection with antimicrobial-resistant bacteria in patients receiving selective digestive decontamination or selective oropharyngeal decontamination could be concluded^[43]. Although there are robust data supporting the effectiveness of different forms of SD controversy persists about the benefit of SID and is extensive to the liver transplant population.

SID in liver transplant patients was introduced by Wiesner *et al.*^[44] in 1988 as a prophylactic strategy against infection. In this study, the incidence of infection following transplantation was markedly reduced by 50%. These investigators postulated that liver transplant recipients constitute a subset of patients that could specially benefit from SID prophylaxis. The fact is that, LTRs make up a relatively homogeneous group of critical care patients with a larger a priori chance of developing infections in comparison with mixed patients in intensive care; therefore, they theoretically should be optimal candidates for SID^[7,44].

Several observational studies^[44-49], randomized clinical trials (RCTs)^[50,51] and a meta-analysis^[52] of SID in liver transplantation have suggested a decrease in post-liver transplantation infection rates with SID, however, other studies have reported no benefit^[28,53-55] (Tables 1 and 2).

Gorensek *et al.*^[47] in a cohort study showed that selective bowel decontamination with a combination of norfloxacin and nystatin was well tolerated and highly

Table 1 Characteristics of randomized trials evaluating selective intestinal decontamination in liver transplant

| Ref. | Type of study | SID regimen | Treatment perioperative (48 h) | Patients (n) | | Patients with infection, n (%) | | Period of observation posttransplantation (d) |
|---|---|---|--------------------------------|--------------|---------|--------------------------------|-----------|---|
| | | | | SID | Control | SID | Control | |
| Bion <i>et al</i> ^[50] , 1994 | Randomized trial not placebo controlled | Tobramycin, amphotericin and polymyxin B for 5-15 d posttransplantation | Cefotaxime and ampicillin | 21 | 31 | 3 (14.3) | 12 (38.7) | 15 d or until hospital discharge |
| Arnrow <i>et al</i> ^[51] , 1996 | Randomized trial not placebo controlled | Gentamicin, polymyxin and nystatin orally for 21 d posttransplantation | Cefotaxime and ampicillin | 36 | 33 | 14 (38.9) | 14 (42.4) | 28 |
| Hellinger <i>et al</i> ^[53] , 2002 | Randomized placebo-controlled trial | Gentamicin, polymyxin E and nystatin 4 x/d for 21 d posttransplantation | Ceftizoxime | 37 | 43 | 12 (32.4) | 12 (28.9) | 60 |
| Zwaveling <i>et al</i> ^[28] , 2002 | Randomized placebo-controlled trial | Norfloxacin, colitin,tobramycin and amphotericin B | Cefotaxime and tobramycin | 29 | 29 | 22 (75.9) | 25 (86.2) | 30 |

SID: Selective intestinal decontamination.

Table 2 Characteristics of observational studies evaluating selective intestinal decontamination in liver transplant

| Ref. | Type of study | SID regimen | Other antibiotics | Patients (n) | | Patients with infection, n (%) | | Period of observation posttransplantation (d) |
|--|---|---|--|--------------|---------|--------------------------------|------------------------|---|
| | | | | SID | Control | SID | Control | |
| Gorensek <i>et al</i> ^[47] , 1993 | Prospective nonrandomized study with historical control | Norfloxacin and nistatin. Oropharyngeal paste (polymyxin, gentamicin, nystatin) | Metronidazole and Third-generation cephalosporins for 5 d | 17 | 34 | 1 (5.9) ¹ | 18 (52.9) ¹ | 30 |
| San-Juan <i>et al</i> ^[54] , 2011 | Prospective cohort study | Fluoroquinolones (norfloxacin or ciprofloxacin) 7 d | First- or second- or third generation cephalosporins, or antipseudomonal beta-lactams or glycopeptides | 415 | 595 | 110 (26.5) | 156 (26.21) | 30 |
| Sun <i>et al</i> ^[49] , 2012 | Retrospective uncontrolled study | Rifaximin | Cefotaxime and ampicillin for 24 h | 30 | 80 | 7 (23.3) | 23 (28.7) | 90 |
| Katchman <i>et al</i> ^[55] , 2014 | Retrospective cohort study | Colitin, gentamicin and nystatin | Cefazolin and metronidazole for 4 d | 111 | 37 | 47 (42.7) | 18 (46.8) | 30 |

¹Only fungal and gram-negative infections reported. SID: Selective intestinal decontamination.

effective. A trend toward better short-term survival in patients receiving SID was also found, but long-term mortality was not different among the treatment and control groups.

Subsequently, Bion *et al*^[50] in a RCT including 52 patients, demonstrated a lower incidence of infections in patients receiving SID compared with the placebo group. The SID regimen was started at the time a donor organ was identified and was extended for 15 d or until hospital discharge.

In 1996, Arnrow *et al*^[51] reported lack of benefit of SID in a clinical trial including 69 patients, although in the subgroup of patients receiving SID for 3 or more days before transplantation it was reported a lower incidence of infection due to aerobic gram-negative bacilli (0% vs 21%, *P* < 0.05) which included intra-abdominal, surgical site, respiratory tract and bloodstream infections.

In a placebo-controlled, double-blind RCT of 80 patients that were followed for the first 60 d after transplant and in which more than 85% of patients received the SID regimen for more than 3 d preoperatively, Hellinger *et al*^[53] reported that there were no statistically significant differences between both groups with regard to infection or mortality.

Zwaveling *et al.*^[28] in a placebo-controlled RCT including 58 patients (with a bacterial or fungal infection rate in the first month posttransplantation of 85% in both groups), no significant protective effects against the development of bacterial infections were found between SID and placebo group. However, the type of microorganism causing infection differed: infections due to Gram-negative bacilli and yeasts were significantly reduced in the group treated with SID. Conversely, infections due to Gram-positive cocci were more prominent among patients undergoing SID, although the difference did not reach statistical significance.

In a systematic review and meta-analysis by Safdar *et al.*^[52] 14 studies analyzing SID in liver transplantation were included (six were uncontrolled studies, four were controlled studies using historical controls and four were RCTs). Only the four RCTs were included in the meta-analysis. Overall, the controlled observational studies showed a reduction in infection with SID (range of RR in treatment groups, 0.09-0.62). Additionally, meta-analysis showed that SID significantly reduced the incidence of infections caused by gram-negative bacteria in the clinical forms of pneumonia and septicemia. However, no reduction was shown in the overall incidence of infection due to an increased incidence of enterococcal infections in patients receiving SID. They did not separately analyze the impact of invasive fungal infections, perhaps due to the low event rate. Neither was evaluated the difference in mortality with the use of SID since the sample size was insufficient.

A further multicenter observational study conducted by San-Juan *et al.*^[54] failed to demonstrate differences in the incidence of early infections between LTR receiving fluoroquinolones for SID (FQ-SID) and those who did not, although SID was related with a relative increase of infections due to multi-resistant gram-negative bacilli suggesting a deleterious effect of SID in terms of selection of antimicrobial resistance.

Rifaximin has also recently been evaluated as SID with non reabsorbable antibiotics in LTR by Sun *et al.*^[49] in an observational study in which the rate of infections in the first 90 d post-transplant was compared between liver transplant recipients who did and did not receive rifaximin for hepatic encephalopathy while being on the waiting list. They found a protective effect of rifaximin against post-transplant infections in the more severely ill liver transplant recipients with no increase in multidrug-resistant bacterial infections.

Finally, a recent observational study failed to demonstrate a reduction in the incidence of early infection by the use of SID in living-donor liver transplant recipients although the application of SID was not related with an ecological impact in terms of the emergence of bacterial resistance^[55].

Several study analyzed *C. difficile* toxin-related diarrhea and it was not recognized any more frequently in patients treated with SID^[49,52,53,55]. Even in

studies where selective bowel decontamination was rifaximin, the risk of *C. difficile* colitis was lower but not statistically different in the treatment group^[49].

The important methodological heterogeneity of all these studies could partially explain the differences in the obtained results. Other crucial aspects that limit interpretation of the results reside on the high variability in the SID regimens used in the different studies either in the type of antimicrobials, the timing of administration (before or after transplantation) and the duration of the treatment.

Nevertheless, available data so far suggest that while the use of SID can be related to a relative decrease of specific infections such as infections due to enterobacteria, it does not seem to globally reduce infection and mortality rates^[28,53], although some experts continue to recommend combined SID strategies (systemic antibiotherapy and topical enteral antibiotherapy) given the high incidence of early bacterial infection in this population and the increased severity of infections caused by *Enterobacteriaceae*, which seem to be prevented to some extent with SID^[7,44].

On the other hand, these strategies seem to be safe as have not been found to promote selection of multiresistant microorganisms in the majority of RCTs evaluating this end point, although all these studies have been conducted in epidemiological contexts of low basal rates of multidrug resistance^[16,21,25,41]. Conversely, in other observational studies performed in areas with high rates of colonization or infection by gram-negative multiresistant^[56,57] or MRSA^[58] selection of these microorganisms have been particularly in relationship with the introduction of SID strategies.

Although the potential risk of selection of resistant microorganisms has conditioned reluctance of the scientific societies to recommend the SID as a preventive strategy, the fact is that whereas systemic administration of antibiotics as part of the SID presumably may favor the selection of microorganisms this deleterious effect is less plausible with the use of topical antibiotics, in which very high antibiotic concentrations are achieved therefore making the emergence of resistance more improbable. In fact, in the few studies of topical SID (oropharyngeal and/or intestinal) in which this problem has been specifically analyzed through directed colonization studies an ecological risk entailed by SID strategy could not be demonstrated^[41].

CONCLUSION

Current available data so far suggest that SID reduces the incidence of gram-negative and yeast infection at the expense of an increased incidence of infections due to some gram-positive microorganisms, generally with less pathogenicity and therefore causing less severe infections. However, none of the studies carried out to date has been able to detect a significant survival benefit probably due to limitations in their sample size.

Anyway, although pooled results trend to be favorable, methodological flaws present in the majority of the studies added to the potential risk of selection of multiresistant microorganisms have conditioned ongoing controversy about the routine use of these strategies. Because of these limitations in the studies conducted to date, randomized controlled studies evaluating SID strategies are needed, preferably analyzing non-absorbable antimicrobials or nonantibiotic products, such as the use of probiotics or prebiotics, which carry a theoretical lower ecological deleterious effect by the low risk for the selection of resistant strains.

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Role of inflammation and infection in the pathogenesis of human acute liver failure: Clinical implications for monitoring and therapy

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Abstract

Acute liver failure is a rare and devastating clinical condition. At present, emergency liver transplantation is the only life-saving therapy in advanced cases, yet the feasibility of transplantation is affected by the presence of systemic inflammation, infection and resultant multi-organ failure. The importance of immune dysregulation and acquisition of infection in the pathogenesis of acute liver failure and its associated complications is now recognised. In this review we discuss current thinking regarding the role of infection and inflammation in the pathogenesis of and outcome in human acute liver failure, the implications for the management of such patients and suggest directions for future research.

Key words: Inflammation; Neuroinflammation; Acute liver failure; Biomarker; Infection

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Core tip: Acute liver failure is a serious and rare condition, for which emergency liver transplantation is the only rescue therapy in advanced cases. The medical need for liver transplantation and feasibility of such an intervention are affected by the presence of systemic inflammation and infection. This review will discuss current thinking with regards to the role of infection and inflammation in the pathogenesis of human acute liver failure, and its effect on outcome. We also provide clinical guidance for the management of these patients and suggest directions for future research.

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and infection in the pathogenesis of human acute liver failure: Clinical implications for monitoring and therapy. *World J Gastroenterol* 2016; 22(26): 5958-5970 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i26/5958.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i26.5958>

INTRODUCTION

Acute liver failure (ALF) is a rare and devastating clinical condition, resulting from massive loss of hepatic parenchyma and metabolic function. ALF is estimated to affect 2000 people per year in the United States, and is associated with a significant mortality rate^[1,2]. The development of hepatic encephalopathy (HE) defines ALF, and confers a poor prognosis. With the use of emergency liver transplantation, up to 75% of patients should now be expected to survive^[3,4]. Acetaminophen (APAP; paracetamol) is the most common cause of ALF in the West, compared with viral causes in the developing world.

Many of the extra-hepatic features of ALF-including hemodynamic disturbance and multi-organ failure- are now thought to be driven by the secondary immune response to hepatocyte cell death. Clinically ALF shares many features with severe sepsis, including a systemic inflammatory response and progression to multi-organ failure. The hemodynamic profile of ALF mirrors that of septic shock, suggesting that the systemic release of inflammatory mediators might be responsible for progression from the inflammatory response to multi-organ failure. Immune dysregulation is now recognised to be central to the pathogenesis of ALF^[5,6] and it is likely that the clinical features and outcomes in ALF relate to an individual patients innate immune response to liver injury, as opposed to the liver injury and hepatocyte cell death itself. As a result of this immune dysregulation, patients with ALF demonstrate increased susceptibility to infection which is associated with the development of further complications. Currently, emergency liver transplantation is the only rescue therapy for patients with advanced ALF, yet the development of infection, sepsis and the resultant inflammatory response and multi-organ failure may preclude the opportunity for life-saving liver transplantation. Novel, non-transplant therapies are urgently needed, and understanding the role of infection and inflammation in the pathogenesis and progression of ALF is central to the development of new therapeutic strategies. Understanding the role and interplay of infection and inflammation in the pathogenesis of ALF will in addition allow better risk stratification and prognostication in ALF. In this review we detail the current thinking with regards to the role of infection and inflammation in the pathogenesis of and outcome in human ALF, their implications for the management of the ALF patient and suggest directions for future research. Comprehensive overviews of

human basic science and research studies undertaken in animal models of ALF are out with the scope of this review.

IMMUNE DYSFUNCTION IN ALF

The immune dysregulation in ALF has been discussed extensively elsewhere^[5-7]. Dysfunction of both the cellular and humoral innate immune system plays a role in the pathophysiological development of ALF. Defective functioning of the cellular components in particular of the innate immune system are implicated in the increased risk of infection in patients with ALF. Causes of innate immune dysfunction are proposed to include changes in gut permeability, endotoxemia, lipoprotein and albumin dysfunction, and toll-like receptor (TLR) expression^[8]. Toll like receptors are innate pattern recognition receptors, present in many cells including neutrophils and hepatocytes. Activation of neutrophil TLR can induce an inflammatory response with phagocytic activity and cytokine release, but whether this is beneficial or detrimental to the patient with ALF is not well defined^[9].

The underlying mechanism of acute liver injury begins with hepatocyte necrosis. The driver of ongoing necrosis in the absence of ongoing injury is not clearly understood. Oxidative stress leads to production of reactive oxygen species, which in turn *via* a cascade of events results in activation of c-Jun N-terminal kinase (JNK). This in turn leads to mitochondrial dysfunction causing further hepatocyte necrosis and release of damage associated molecular patterns (DAMPs). DAMPS activate hepatic macrophages and as a further consequence the inflammasome is formed. Comprehensive reviews of the role of inflammasomes in liver disease are available elsewhere^[10,11]. Briefly, inflammasomes are multiprotein complexes that sense intracellular danger signals *via* NOD-like receptors. The inflammasome finely controls the inflammatory response, by responding to low-threshold signals. Activation of the inflammasome by DAMPS is a result of TLR activation and activation by an inflammasome ligand, and results in caspase-1 activation and IL-1 β secretion - see Figure 1. The NLRP3 inflammasome is the most well characterised member of the inflammasome family and three potential activation pathways have been proposed: (1) extracellular ATP signal resulting in potassium efflux and pannexin recruitment; (2) endocytosis of crystallized cholesterol, uric acid or amyloid with lysosomal damage following phagocytosis of these particles; and (3) activation by reactive oxygen species. Looking specifically at the role of the inflammasome in acute liver failure, inflammasome activation in APAP-ALF has been studied^[11]. Necrotic hepatocytes and sinusoidal endothelial cells release DAMPS, which activate the inflammasome as described above. It remains unclear as to whether APAP directly results in inflammasome

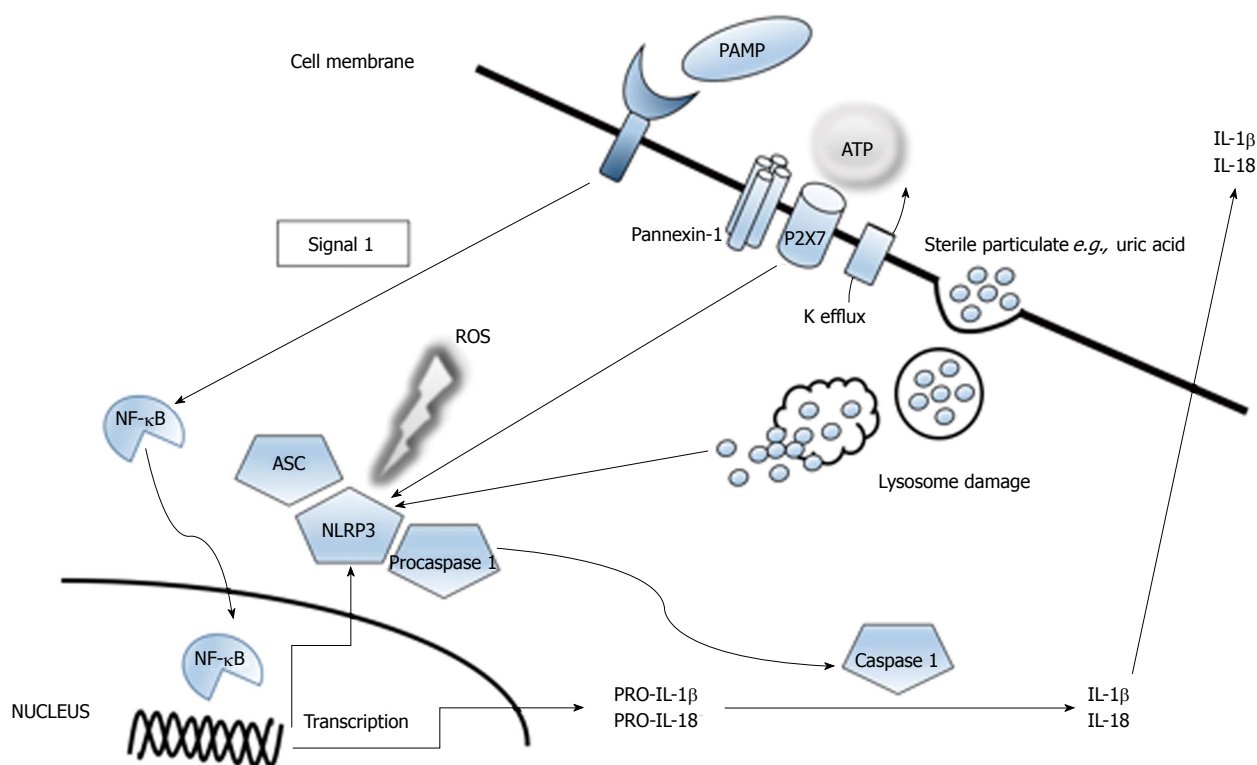


Figure 1 Activation of the NLRP3 inflammasome. NLRP3 inflammasome activation. Extracellular ATP is sensed by the P2X7 purinergic receptor and results in potassium efflux and recruitment of pannexin. Activation may also be induced by large particles such as uric acid, and lysosomal damage after phagocytosis of these particles induces NLRP3 inflammasome activation. Reactive oxygen species also contribute to inflammasome activation. Activation of the inflammasome results in caspase-1 and IL-1 β secretion.

formation. The inflammasome is a determinant of hepatic inflammation in APAP-ALF, but its role in regeneration - critical in determining the outcome of the patient - is uncertain. Much of the research undertaken looking at the role of the inflammasome in APAP-ALF has been undertaken in mouse models, and therefore may not be directly applicable to human clinical practice.

Subsequently, pro-inflammatory cytokines are released, leading to the recruitment of more immune cells to the site of inflammation and further advancing hepatocyte cell death. The roles of specific cell types are discussed briefly below and highlighted in more detail in Table 1^[12-20]. A comprehensive overview of other pathophysiological mechanisms important in modulating inflammation and infection in ALF such as autophagic dysfunction, mitochondrial membrane potential dysregulation and the influence of calcium flux are out with the scope of this clinical review, but are extensively reviewed elsewhere^[21-23].

Neutrophils

Neutrophils - a major subset of innate immune cells - are rapidly recruited to the liver in response to liver injury. With the evidence available at present, it is unclear as to whether neutrophils directly potentiate liver injury^[12]. Locally, neutrophils become activated by cytokines which may result in progressive tissue damage *via* release of proteolytic enzymes

and reactive oxygen species. In severe sepsis, a condition which shares many clinical features with ALF, systemic neutrophil activation is associated with a functional immune paresis. Neutrophils assist in removal of necrotic cell debris in preparation for tissue repair and resolution of the inflammatory response. In ALF, neutrophils have decreased phagocytic activity - see Table 1. This decrease in phagocytosis correlates with arterial ammonia concentration, which has been shown in several studies to predict the development of cerebral edema in advanced hepatic encephalopathy^[24,25]. In summary, circulating neutrophils in ALF appear to have impaired bactericidal function, and this is likely to be relevant in the increased susceptibility to infection, which may subsequently preclude life-saving liver transplantation, and hence a complete understanding of the role of cells involved in fighting infection such as neutrophils is essential.

Monocytes and macrophages

Monocytes and hepatic macrophages mediate the inflammatory response and tissue repair process occurring in ALF. Kupffer cells - resident hepatic macrophages - potentiate liver injury by sensing DAMPs and releasing pro- and anti-inflammatory mediators (with TNF- α being relevant in sensitizing hepatocytes to apoptosis); however the extent of their role remains incompletely understood^[7]. Hepatic macrophages

Table 1 Cell types and proteins involved in immune pathogenesis of acute liver failure

| Ref. | Cell type/protein study population | Finding in ALF |
|--|--|--|
| Taylor <i>et al</i> ^[12] | Neutrophils ALF | Significant reduction in neutrophil surface expression of CD16 (causing reduced binding capacity) and neutrophil phagocytic activity compared with healthy controls. Impaired phagocytic activity predictive of death without transplantation |
| Manakkat Vijay <i>et al</i> ^[13] | Neutrophils APAP-ALF | Toll-like receptors sense pathogens and induce inflammatory responses. Neutrophil toll-like receptor 9 expression increased on day 1 compared with healthy controls, and correlated with severity of HE and SIRS |
| Srungaram <i>et al</i> ^[14] | Osteopontin (activates neutrophils and macrophages) | Median osteopontin levels significantly elevated in the ALF group compared with comparator cohorts; median osteopontin levels were highest in patients with APAP-ALF and ischaemic hepatitis (conditions associated with a hyperacute course and better outcomes - osteopontin may have a central role to play in the resolution of ALF) |
| ALFSG Lawson <i>et al</i> ^[15] | Neutrophils APAP-ALF | Neutrophils accumulate in liver parallel to or slightly after liver injury; number of neutrophils in liver substantial compared with baseline, with increased levels of TNF- α , KC and MIP-2 chemokines |
| Sehgal <i>et al</i> ^[16] | Monocyte-macrophages Hepatitis E in Pregnancy | Functionality of monocytes and macrophages impaired in pregnant patients developing ALF compared with those with ALI. |
| Wigmore <i>et al</i> ^[17] | Monocytes ALF | Peripheral blood mononuclear cells from patients with ALF show reduced potential to produce IL-6 and TNF and elicit an acute phase response <i>in vitro</i> |
| Leifeld <i>et al</i> ^[18] | Macrophages ALF | CC-chemokines recruit and activate macrophages and T-cells. Elevated levels of serum and intrahepatic chemokines in ALF compared with controls, correlating with extent of infiltration by macrophages and T-cells. |
| dos Santos <i>et al</i> ^[19] | Eosinophils ALF | High number of intrahepatic eosinophils in ALF, associated with increased expression of IL-6. |
| Wyke <i>et al</i> ^[20] | Complement system ALF | Defective opsonisation due to complement deficiency. Complement factors reduced to below 40% of the activity of control serum |

ALF: Acute liver failure; APAP: Acetaminophen; SIRS: Systemic inflammatory response.

demonstrate plasticity, with their function extending from pro-inflammatory to pro-resolution. There is evidence for tolerance of circulating monocytes to bacterial endotoxins, which further impedes host immunity^[26]. Liver regeneration following injury requires functional liver macrophages, the numbers of which are controlled by CSF-1 (macrophage colony stimulating factor). In patients with APAP-ALF, low serum levels of CSF-1 were associated with increased mortality^[27]. Of note, administration of CSF-1 to mice increased innate immunity^[27], raising the possibility that CSF-1 could be developed as a potential therapeutic target in humans. Investigating further the relationship between neutrophils, macrophages and other immune cells in human ALF would provide a clearer understanding of the pathophysiology of this condition, and potentially provide a target for urgently needed new therapies.

INFECTION AND ALF

Patients with ALF have increased susceptibility to both bacterial and fungal infection as a result of a conglomerate of factors, including reduced complement levels, impaired phagocytic function, and the need for invasive interventions. In addition, for example in those patients with cerebral edema, actions usually taken to reduce infection risk such as chest physiotherapy are contra-indicated due to the risk of exacerbating intracranial hypertension (ICH). Infection usually develops early in the course of ALF,

with a median onset of 2-5 d from admission^[28]. More recently, infection has been reported as a later complication in ALF, occurring at a median of 10 d^[29]. Importantly, the presence of bacterial or fungal infection may preclude listing for OLT, at present the only curative option for advanced ALF.

As such, the development of infection has significant prognostic implications. There is a complex and as yet not completely understood relationship between infection, hepatic encephalopathy (HE) and outcome. Rolando demonstrated that infection is more frequent in those with higher grades of HE and higher SIRS (systemic inflammatory response) score, as a result of the mechanisms described above^[30]. It is often difficult however to tease out what is cause and effect in the relationship between HE and infection. Other groups have also reported a link between the acquisition of infection and both the development and progression of HE^[31]. Perhaps unsurprisingly, infection is a leading cause of death in ALF and is responsible for late death in at least a quarter of cases^[28,32]. Despite this, the development of bacteremia has not been consistently shown to be independently predictive of mortality^[29]. Predictors of bacteremia in ALF include admission HE grade > 2, maximum HE grade and admission SIRS score > 1^[29]. The presence of a SIRS response may in fact reflect the presence of subclinical infection, or may reflect the development of an inflammatory and subsequent anti-inflammatory response, which as discussed later in this review may predispose to the acquisition of infection.

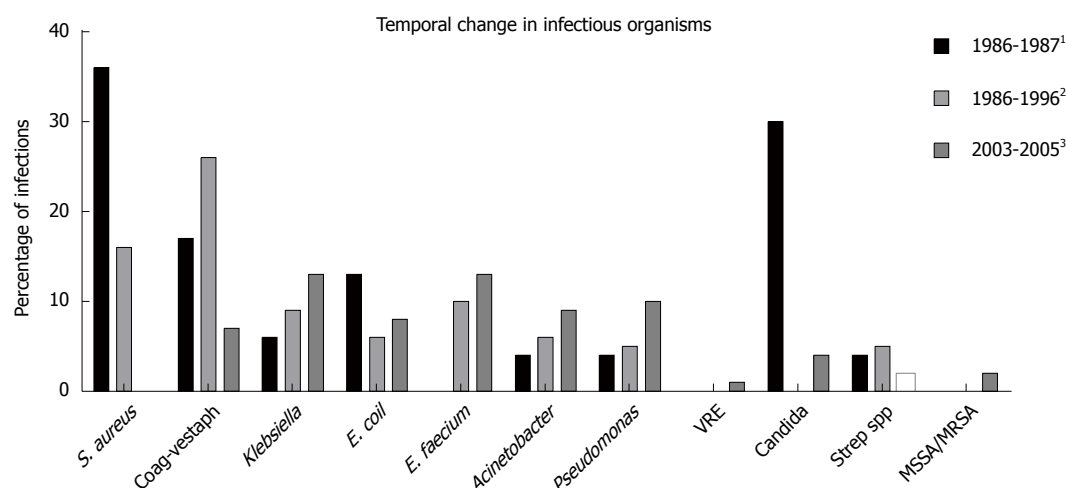


Figure 2 Temporal change in infections in acute liver failure. ¹Adapted from Rolando *et al*^[28], ²Adapted from Wade *et al*^[37], ³Adapted from Karvellas *et al*^[29].

In addition, some therapies used to treat specific etiologies of ALF may also lead to the development of infectious complications, and such treatments must be used with caution when there is evidence to suggest that the development of infection is associated with a poorer outcome. One Japanese group studied the development of infectious complications in patients with autoimmune ALF treated with corticosteroids; corticosteroids were given to 19 patients, and 17 infectious complications were identified in 12 patients, at a median of 15 d^[33]. Importantly, no significant differences in the clinical or biochemical features of the patients with and without infection were noted^[33], reinforcing the need for a high index of clinical suspicion for infection in this group of patients. Another group reported opportunistic infection in 21.6% of ALF patients receiving steroid therapy, with cytomegalovirus and *Pneumocystis jiroveci* being most commonly implicated^[34].

Diagnosis of infection in ALF

In patients with ALF, the clinical signs of infection such as pyrexia and elevated peripheral white cell count are absent in up to 30% of patients^[35], and clinical suspicion must remain high in patients with a deteriorating course. In addition, the hemodynamic profile in ALF is similar to that observed in septic shock, adding an extra level of complexity in distinguishing between the two conditions. C-reactive protein (CRP) - a commonly used marker of infection in other clinical situations - is unhelpful in the patient with ALF. CRP is produced exclusively by hepatocytes and therefore low levels are often measured in ALF consequent to reduced hepatic parenchyma. A low CRP therefore does not reflect lack of significant inflammation or infection; Silvestre studied 7 patients with ALF and sepsis and in all septic patients, CRP levels were markedly decreased and on occasions undetectable^[36], confirming the futility of CRP as a marker of infection in ALF. When meticulous microbiological surveillance

is undertaken, clinical or bacteriological evidence of infection is found in up to 90% of patients with ALF^[28]. Another difficulty in characterizing infections and the effect of antimicrobials in patients with ALF admitted to tertiary liver centres is that many patients are transferred from other units where they may have had microbiological cultures performed and received anti-microbial therapy. Often this information is not available and these factors may skew the results of studies of infection undertaken in tertiary liver centres.

Bacterial infection

Bacterial infections are most common, documented in 30%-80% of ALF patients^[28,35,37]. Since the description of ALF as a disease entity, both the timing of the development of bacteremia and the organisms isolated has changed (Figure 2)^[28,29,37]. Older data suggested that bacteremia was an early complication of ALF, occurring at a median of 3 d^[28]. More recent data now support the development of bacteremia as a late complication, with Karvellas reporting a median time to bacteremia of 10 d^[29]. Gram positive bacteremia was previously most common, reported in 73% of those with confirmed blood stream infection^[37]. However, Gram negative bacteremia is now more common, identified in 52% of the Karvellas cohort^[29]. This is of real clinical importance, as at least one study has identified a trend towards progression of HE in patients with gram negative infection compared with gram positive infection^[31]. In general, critically ill patients are at increased risk of infection with antibiotic-resistant organisms such as vancomycin resistant enterococcus (VRE) and methicillin resistant *Staphylococcus aureus* (MRSA). Older reports of infection in ALF identified that pneumonia was the most common infection in ALF, accounting for 50% of all infections^[28,35]. Urinary tract infections were the second most frequent, accounting for 22% of infections^[28]. Gram positive bacteria were most commonly isolated and frequently related to pulmonary sepsis. In a more recent study of patients

with ALF admitted to a Liver Intensive Care Unit, 35% developed bacteremia^[29]. The most frequently isolated pathogens were *Enterococcus faecium*, *Klebsiella* spp and vancomycin-resistant *Enterococcus*^[29], highlighting a shift in organisms most commonly implicated in bacterial infection in ALF. This may reflect changes in the choice of antimicrobial prophylaxis regimens over time, selecting out particular and resistant organisms.

Late deaths (> 7 d) in patients with ALF are often attributable to superadded bacterial infection; Rolando reported that in their cohort, all deaths occurring 7 d after admission were directly related to bacterial infection^[28], highlighting the importance of remaining vigilant for the development of bacterial infection, particularly later in the course of the illness.

Fungal infection

Fungal infections are also common in the ALF patient cohort, occurring in around one third^[38]. *Candida* species is most frequently isolated, and affected patients commonly have concurrent bacterial infection^[38]. *Aspergillus* sp. and *Pneumocystis jiroveci* are also recognised to be opportunistic fungal infections in ALF^[34,39]. Aggressive investigation for fungal infection should commence in the patient with deterioration in HE grade, persistent pyrexia, renal failure or a markedly elevated white cell count, in particular if the patient is already receiving and deteriorating despite broad spectrum antibiotics^[38]. Without treatment, mortality with fungal infection is up to 100%^[38], suggesting a possible role for prophylactic antifungal therapy. To date, no study has looked specifically and solely at the role of prophylactic antifungal therapy in ALF, and such a study may be challenging to undertake as the majority of patients will have concurrent bacterial infection which may confound results.

Viral infection

Opportunistic viral infections are common in all critically ill patients due to functional immunosuppression. In a cohort of critically ill patients due to a variety of etiologies requiring admission to the intensive care unit, cytomegalovirus (CMV) reactivation occurred in 33%, with CMV infection at any level being independently associated with death at 30 d^[40]. In the ALF cohort, viral infection appears to be particularly frequent in those patients receiving steroid therapy. One study reported that of patients who were receiving steroid therapy in ALF, 25.8% developed CMV infection^[34], highlighting the importance of considering the development of viral infections in appropriate patients, particularly in those who are immunosuppressed. However, the clinical relevance of opportunistic viral infection was not discussed in this study, and its impact upon outcomes remains an area for future study.

Antimicrobial prophylaxis

Despite the plausible rationale behind giving prophylactic antimicrobials to reduce risk of infection and the associated impact on HE and outcome, there is no published evidence to confirm that this approach results in a clear mortality benefit. The Acute Liver Failure Study Group (ALFSG) retrospectively assessed the impact of antimicrobial prophylaxis (physician dependent and including both anti-bacterial and anti-fungal agents) on rates of blood stream infection (BSI) and 21-d survival in ALF^[32]. Of a cohort of 1551 patients with ALF (the most common etiology being APAP), 34% had at least one episode of culture-positive infection and 14.6% had at least one episode of BSI. 39% of all patients received antimicrobial prophylaxis; 19% of this cohort received antifungal therapy. Those patients receiving prophylaxis had higher HE grade and had a higher requirement for organ support, generally reflecting a sicker group of patients requiring more invasive therapy, and therefore potentially at higher risk of infection. However, there was no significant difference in the probability of developing BSI in patients receiving prophylaxis compared with those without prophylaxis ($P = 0.12$). In the APAP subgroup, patients receiving prophylaxis were more likely to proceed to transplantation, but there was no significant difference in overall 21-d survival. Looking at the whole cohort on multivariate analysis, antimicrobial prophylaxis did not confer a benefit on 21-d survival. It must also be borne in mind that as discussed above, empirical use of antibiotics may lead to the development of multidrug resistant organisms.

Changes in gut permeability may contribute to the development of infection in ALF, and therefore it has been proposed that selective gut decontamination with poorly absorbable oral antibiotics may be an effective method to reduce the risk of bacterial translocation and infection in ALF. Salmeron reported that in a small group of patients, the administration of poorly absorbable oral antibiotics significantly reduced the likelihood of developing infection; this reduction was predominantly related to a difference in the rate of infection from enterobacteria^[41]. The clinical relevance of infection with this specific organism is not clear, and the routine use of selective intestinal decontamination cannot be advocated at present.

In summary, data regarding antimicrobial prophylaxis in ALF are limited. The ALFSG recommend that prophylactic antimicrobials and antifungals cannot be advocated in all patients - particularly those with mild HE - as these have not been consistently shown to improve overall survival rates^[42]. The ALFSG advise that periodic surveillance cultures are undertaken and therapy initiated promptly according to culture results at the earliest indication of active infection or clinical

deterioration^[42]. Many units therefore commence antimicrobials at the development of higher grade HE requiring intubation and ventilation, the development of SIRS or otherwise unexplained clinical deterioration.

Infection and outcome

Rolando has reported upon the impact of BSI in ALF, with an attributable mortality of up to 60%-76%^[28,30]. Late deaths (*i.e.*, greater than 7 d after admission) may all be attributable to the acquisition of infection^[28]. Karvellas recently demonstrated a significant increase in 21-d mortality in patients developing BSI, and this risk was higher in those patients with non-APAP etiology^[32]. This is likely related to the often sub-acute and prolonged course of this illness and development of the compensatory anti-inflammatory response, both of which result in predisposition to infection. However, in an earlier study, Karvellas failed to demonstrate a significant association between the development of bacteremia and poorer outcome^[29]. The data regarding the association between infection and outcome in ALF are therefore somewhat conflicting, however clinically and pathophysiologically it is logical that the acquisition of infection results in a deteriorating course and poorer outcome.

SIRS

SIRS is the result of a clinical response to an insult of infectious or non-infectious origin, and occurs as a result of systemic pro-inflammatory (*e.g.*, TNF- α , IL-1, IL-6) and anti-inflammatory (*e.g.*, IL-10) cytokine release. The source of this systemic cytokine release may be from hepatocyte cell death and the necrotic liver itself, secondary to endotoxemia or impaired hepatic cytokine metabolism^[43-45]. SIRS is defined as two or more of: temperature $< 36^{\circ}\text{C}$ or $> 38^{\circ}\text{C}$, heart rate > 90 beats/min, leucocyte count $< 4 \times 10^9/\text{L}$ or $> 12 \times 10^9/\text{L}$ and tachypnea > 20 breaths/min or $\text{PaCO}_2 < 4.3$ kPa. In a general population of acutely ill hospitalized patients, patients with SIRS response had a 6.9 times higher 28-d mortality than those without SIRS, and the severity of SIRS correlated with the severity of organ dysfunction and mortality rate^[46]. In ALF specifically, SIRS is believed to be an important factor in the development of renal impairment, HE, multi-organ failure and death.

Difficulties in utilizing SIRS in studies of ALF

In studies of SIRS in patients with ALF, the respiratory component is often not included as many patients are ventilated and accepted respiratory parameters set. However, this approach is not uniform and not always commented upon in study methodology; this must be borne in mind when analyzing the results of any study assessing the utility of the SIRS score in ALF. In addition, many studies assess SIRS at a single time point only, whereas the true value of the score

may come with serial measurements and assessment of trends. Furthermore, the appropriate cut off SIRS score for predicting prognosis in ALF has not been defined. Therefore there are limitations currently in using the SIRS score to predict prognosis in ALF, and further work is required before this score can be accepted as a prognostic marker in isolation, or be included in other validated prognostic models.

Development of SIRS and CARS

Local tissue injury is the initiating factor in the development of SIRS, and this can arise from any number of insults in the setting of ALF. Initially, this injury triggers a marked release of a number of pro-inflammatory mediators (*e.g.*, DAMPS, TNF- α , IL-6 and IL-8)^[5] through the activation of macrophages, polymorphonuclear cells, endothelial cells and the complement system. These pro-inflammatory mediators are now recognized to be associated with both tissue regeneration and tissue damage. Circulating monocytes and lymphocytes are attracted to the area of injury, and act to increase the local response to injury. Platelet and coagulation cascade activation occurs with an increase in vascular permeability and spill over of pro-inflammatory mediators, which may in turn lead to the SIRS. Systemic pro-inflammatory cytokine release following local tissue injury subsequently results in an increase in the level of circulating anti-inflammatory mediators. The point at which and the stimulus for the switch to the production of anti-inflammatory mediators is not well defined, but this counter response aims to prevent overwhelming systemic inflammation. This compensatory anti-inflammatory response syndrome (CARS) is associated with raised levels of anti-inflammatory mediators (IL-4, IL-10, TGF- β), and an impairment in cellular immune function^[5]. Monocyte deactivation is key in the development of CARS^[47]. This CARS phase results in an increased risk of and decreased clearance of infection, and multiorgan failure^[5].

The development of SIRS is associated with poorer outcomes. SIRS is associated with progression of HE^[31,48], development of bacteremia^[29] and in some studies a poorer outcome independent of the presence of infection^[48,49]. Karvellas reported that on multivariate analysis, SIRS was not predictive of mortality either in ALF patients (all etiologies) developing bacteremia or in non-transplanted ALF patients^[29]. In contrast, Craig demonstrated that SIRS occurred significantly earlier and was of a greater magnitude in patients with APAP-ALF who died compared with patients who survived, with the number of SIRS components fulfilled post overdose being significantly associated with increased mortality at 48, 72 and 96 h time points^[49]. These data suggest that perhaps the clinical effect of developing SIRS is etiology specific. SIRS develops more frequently in APAP-ALF compared with non-APAP ALF^[50], and this may reflect the

hyperacute course of APAP-ALF. The role of SIRS in non-APAP ALF is less well defined and is an area worthy of future research. Analysing all etiologies of ALF, Leithhead identified that the presence of SIRS was associated with a reduced chance of spontaneous survival^[50]. Miyake *et al.*^[48] assessed the relationship between SIRS and outcome in 99 patients with non-APAP ALF. In this cohort, increasing SIRS score was significantly associated with the development of adult respiratory distress syndrome (ARDS), disseminated intravascular coagulation (DIC), acute renal failure and multi-organ failure. Patients with a SIRS score of 0 or 1 had a significantly higher chance of survival than patients with a SIRS score of 2. This group also demonstrated - albeit in small numbers - that using SIRS as a predictor of prognosis had better specificity and positive predictive values when compared with the Kings College Hospital Poor Prognostic Criteria (KCC)^[48]. The same group analyzed the association between SIRS and outcome in fulminant hepatitis B and reported that a SIRS response was independently associated with 1 wk and overall mortality^[51]. The role of SIRS in the development of HE and renal dysfunction will be discussed further below.

NEUROINFLAMMATION AND HEPATIC ENCEPHALOPATHY

Progression of HE is a major determinant of outcome in ALF, and increasingly the presence of infection and/or a SIRS response is recognised to predispose to increasing HE grade and a poorer prognosis^[30,31]. Following progression, patients with deeper HE are at greater risk of developing infection, as a result of poorer liver function and the invasive interventions required to manage their condition.

Key mechanisms in the development of central nervous system (CNS) complications of ALF, including cerebral edema, are incompletely understood, and much of the basic research has been undertaken in animal models of ALF. Raised arterial and brain ammonia levels - as a consequence of decreased removal by the liver - have been shown to correlate with the severity of CNS complications in ALF^[52]. Ammonia sensitizes macrophages to activating stimuli, increasing the secretion of pro-inflammatory cytokines, thereby perpetuating the progression of HE and cerebral edema, yet the use of ammonia lowering therapies have not been consistently proven to prevent or treat such complications^[53,54].

The concept of the "inflamed brain" in ALF has been proposed^[55], and TNF- α , IL-1 β and IL-6 are thought to be key mediators in this process. Neuro-inflammation may occur as a result of a direct effect of systemically derived pro-inflammatory cytokines and/or effects of local cerebral accumulation of lactate which subsequently causes a direct neuroinflammatory response^[56]. Neuro-inflammation is characterized

by microglial activation and local release of pro-inflammatory cytokines. Increased cerebral lactate levels correlate with microglial activation and cerebral pro-inflammatory cytokine production, confirming its importance in the pathophysiology of HE^[57]. TNF- α levels have been shown to be elevated in relation to the severity of HE in ALF^[58]. Furthermore, TNF- α gene polymorphisms influence outcome in human ALF, and in the setting of APAP-ALF, decreased TNF- α production appears to protect against the development of severe HE^[59].

There has been some debate as to whether the blood-brain barrier (BBB) remains intact in ALF^[56,60]. Both permeability changes and complete breakdown of the BBB have been suggested. In human ALF, studies have failed to demonstrate structural BBB breakdown in contrast to the findings in murine studies^[61,62]. However, TNF- α is known to disrupt the BBB and in septic encephalopathy structural BBB breakdown is reported^[63], therefore it seems likely that some degree or form of BBB breakdown is intrinsically involved in the pathogenesis of HE in ALF. Transfer of systemic pro-inflammatory cytokines across a disrupted blood-brain barrier is one proposed mechanism for the development of CNS complications. Further work is required to establish the role of the blood-brain barrier - and its particular relationship with TNF- α in the pathogenesis of HE in human ALF, to allow the development of targeted therapies.

In ALF, a significant correlation is seen between both the presence and severity of systemic inflammation, and the development of CNS complications, in particular intracranial hypertension (ICH)^[51,52]. This appears to be particularly relevant in cases of APAP-ALF and in non-infected patients, a higher number of SIRS components present at admission (reflecting either sterile inflammation or occult infection) was associated with a progression to deeper stage HE^[31]. In patients requiring intervention for raised intracranial pressure (ICP), SIRS score and levels of IL-1 β , TNF- α and IL-6 were significantly higher than in patients not requiring therapy for raised ICP^[52]. TNF- α levels correlated with cerebral blood flow (rapid increases in which are proposed to underlie the development of raised ICP in ALF)^[52]. Furthermore, in the group of patients who did not require specific therapy for raised ICP, increases in the number of SIRS components fulfilled, CRP and TNF- α were associated with the development of surges of increased ICP^[52]. These findings confirm that both systemic and local inflammation is important in the pathogenesis of the raised ICP of ALF. The acquisition of infection (with or without the development of SIRS) during grade I-II HE has been shown to be predictive of worsening HE in a group of patients with APAP-ALF, but interestingly not in patients with non-APAP ALF^[31]. Despite this, Vaquero reported that detection of infection during the early stage of HE was not associated with a poorer outcome^[31]. These findings

raise the possibility that prophylactic antibiotics might benefit patients with APAP-ALF and early HE.

Recognition of the role of neuroinflammation and infection in the progression of HE allows the development of pathophysiologically based management strategies. As infection and SIRS appear to be intrinsically linked to the development and progression of HE, stringent microbiological surveillance and prompt treatment of infection are a cornerstone in management of HE in ALF. With regards to novel therapies, as TNF- α has been demonstrated to be involved in the development of HE, the use of albumin dialysis to facilitate removal of TNF- α has been proposed^[64]. In animal models of ALF, etanercept (an anti TNF- α molecule) prevents microglial activation and IL-6 accumulation, delaying the onset of HE and cerebral edema^[65]. It may be that etanercept provides a new therapeutic target for the patient with ALF, but primarily the role of TNF- α and related genetic polymorphisms in human ALF needs to be confirmed.

Other novel therapies targeting neuroinflammation in ALF include N-acetylcysteine (NAC) and minocycline. NAC crosses the BBB, and is reported to have anti-inflammatory properties^[66]. NAC presumably has both peripheral and central actions, and has been shown to slow progression of HE and prevent cerebral edema^[67,68]. Minocycline is a tetracycline antibiotic that appears to prevent microglial activation, which is known to be a major factor in the development of HE^[69]. The role of indomethacin - a cyclo-oxygenase inhibitor - has also been studied; it appears to prevent the effects of cytokines on cerebrovascular endothelial cells, which form the BBB^[70]. The latter two therapies have been studied predominantly in animal models of ALF, and as yet their therapeutic benefit has not been translated to human practice.

INFECTION, INFLAMMATION AND RENAL DYSFUNCTION

Renal failure is a common complication of ALF (more common in APAP-ALF than non-APAP ALF, at least in part due to direct renal toxicity caused by APAP)^[50], and is associated with increased mortality^[71]. Despite the clear prognostic importance of the development of renal dysfunction, the pathogenesis is not well understood. Some schools of thought believe that the renal dysfunction of ALF shares similar pathophysiological mechanisms to the hepatorenal syndrome of chronic liver disease^[72]. As SIRS has been shown to contribute to the development and progression of HE and multiorgan failure, it has been proposed that the SIRS response may also be implicated in the specific development of renal dysfunction in ALF. The systemic inflammatory response stimulates apoptotic death of renal tubular cells^[73], and both TNF- α and IL-6 contribute to hemodynamic disturbance in ALF, which may lead to pre-renal acute kidney injury (AKI)^[74].

Studying a cohort of 308 patients with ALF, Leithead

reported that 70% of patients demonstrated a SIRS response; overall, SIRS was more prevalent in the APAP-ALF cohort and was not affected by the presence of infection^[50]. Those patients with APAP-ALF were more likely to develop renal dysfunction (as defined by the RIFLE criteria) than those with non-APAP ALF and this likely relates in part to a direct nephrotoxic effect of acetaminophen. Overall, patients who developed renal dysfunction demonstrated a greater systemic inflammatory response, with an increasing number of SIRS components fulfilled being associated with an increased probability of renal dysfunction. 78% of AKI patients developed SIRS, compared with 53% of the non-AKI patients and on multivariate analysis SIRS remained an independent risk factor for the development of AKI^[50]. Patients with renal dysfunction were also more likely to demonstrate infection and superadded infection was associated with development of AKI; in both those with and without infection, SIRS was more common in the AKI patients. The risk of developing AKI and the impact of SIRS is different in the APAP-ALF and non APAP-ALF cohorts. In APAP-ALF, patients developing AKI were not more likely to have a SIRS response, and they were not more likely to have infection. In contrast, in non-APAP ALF there was a strong association between SIRS and renal dysfunction^[50].

The relationship between pro-inflammatory mediators, SIRS and renal dysfunction in ALF suggest that the inflammatory cascade is central to the development of renal dysfunction. Therapies which target this inflammatory response may therefore be vital in preventing the development and halting the progression of AKI in ALF, affording a subsequent mortality benefit.

BIOMARKERS OF INFECTION AND INFLAMMATION IN ALF

There remains an urgent need for dynamic, easy to apply ALF- specific biomarkers that can be used for predicting prognosis in ALF, as currently applied prognostic scoring systems fall short on negative predictive value and specificity. Recently, a multitude of biomarkers reflecting the important role of inflammation and infection in the pathogenesis and prognosis of ALF have been proposed and studied- see Table 2^[75-80]. The majority of biomarkers described in the table remain research tools at present. However, the neutrophil-lymphocyte ratio (NLR) has been proposed as an important marker of systemic inflammation. This marker is rapidly available and cost effective as it can be calculated from routine laboratory tests. The NLR has been studied in other inflammatory conditions, and found to be of prognostic value^[81,82]. In ALF, NLR has been studied in single time point and staggered APAP overdose^[83]. Craig reported that median NLR was higher at 72 and 96 h post

Table 2 Proposed biomarkers in acute liver failure with an immune basis

| Ref. | Biomarker study population | Relevance | Finding in ALF |
|--|---|---|--|
| Antoniades <i>et al</i> ^[75] | Monocyte HLA-DR expression APAP-ALI | Monocytes are key in the immune dysregulation of ALF | Percentage of monocyte HLA-DR expression significantly lower in ALF patients compared with healthy controls, correlating with poor prognosis |
| Koch <i>et al</i> ^[76] | Soluble urokinase plasminogen activator receptor (suPAR) ALF (all aetiologies) | suPAR related to immune activation in systemic inflammation | suPAR levels significantly increased in ALF patients, correlating with parameters reflecting hepatocyte injury |
| Craig <i>et al</i> ^[77] | Pentraxin-3 APAP-ALI | Pentraxins are soluble pattern recognition receptors forming part of the humoral innate immune system | Admission levels of pentraxin-3 significantly higher in patients with APAP-ALI than those with non-APAP ALI. Pentraxin-3 levels significantly higher in APAP-ALI patients who died/required transplantation <i>vs</i> spontaneous survivors. |
| Rule <i>et al</i> ^[78] ALFSG | Procalcitonin ALF (all aetiologies) | Biomarker of bacterial infection studied in other clinical conditions | No difference in procalcitonin levels in pre-defined severity groups, non-SIRS and SIRS groups with no documented infection and no correlation with presence of infection |
| Antoniades <i>et al</i> ^[79] | Secretory Leukocyte Protease Inhibitor APAP-ALI | Stimulates epithelial cell proliferation and modulates macrophage function | Higher SLPI levels were associated with a greater liver injury, infection and adverse outcome |
| Craig <i>et al</i> ^[80] | Neopterin and soluble CD163 (sCD163) APAP-ALI | Markers of macrophage activation | Levels of both markers significantly higher in APAP-ALI compared with CLD and healthy controls. No association between biomarker level and presence of infection. |

ALF: Acute liver failure; APAP: Acetaminophen.

overdose in single time point overdoses in patients who subsequently either died or were transplanted compared with those who spontaneously survived. A NLR > 16.7 during first 96 h following overdose correlated with the development of HE. Interestingly however, in the staggered overdose cohort, NLR was not predictive of adverse outcomes either at admission or subsequently^[83]. Future human research studies should make identification and validation of a pathophysiologically based biomarker a priority, to assist in decision making regarding need for urgent liver transplantation.

REMAINING PROBLEMS AND FUTURE RESEARCH DIRECTIONS

A better understanding of the pathophysiology of ALF- including the individual components involved in immune dysregulation and the role of infection- is vital to the development of novel treatments. Increasing understanding of the underlying pathophysiological mechanisms of acute liver injury and resolution in mouse models gives rise to the hope of developing new treatment options in humans, in whom currently liver transplantation is the only curative treatment, yet limited by organ availability and patient suitability. Monocyte and macrophage numbers and function may be the key and should be a priority focus in human research. Targeting cytokines proven to play a role in human ALF is also pivotal. However, some concern has also been raised about trialling such therapies due to a potential risk of infection, in patients who are already at higher risk of acquiring bacterial or fungal infection. Much work needs to be undertaken in the

field of immunology and infection before ALF becomes a treatable disease without the requirement for organ transplantation.

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Pancreatic cancer stem cell markers and exosomes - the incentive push

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and incidence is increasing. Poor prognosis is due to late diagnosis and early metastatic spread, which is ascribed to a minor population of so called cancer stem cells (CSC) within the mass of the primary tumor. CSC are defined by biological features, which they share with adult stem cells like longevity, rare cell division, the capacity for self renewal, differentiation, drug resistance and the requirement for a niche. CSC can also be identified by sets of markers, which for pancreatic CSC (Pa-CSC) include CD44v6, c-Met, Tspan8, alpha6beta4, CXCR4, CD133, EpCAM and claudin7. The functional relevance of CSC markers is still disputed. We hypothesize that Pa-CSC markers play a decisive role in tumor progression. This is fostered by the location in glycolipid-enriched membrane domains, which function as signaling platform and support connectivity of the individual Pa-CSC markers. Outside-in signaling supports apoptosis resistance, stem cell gene expression and tumor suppressor gene repression as well as miRNA transcription and silencing. Pa-CSC markers also contribute to motility and invasiveness. By ligand binding host cells are triggered towards creating a milieu supporting Pa-CSC maintenance. Furthermore, CSC markers contribute to the generation, loading and delivery of exosomes, whereby CSC gain the capacity for a cell-cell contact independent crosstalk with the host and neighboring non-CSC. This allows Pa-CSC exosomes (TEX) to reprogram neighboring non-CSC towards epithelial mesenchymal transition and to stimulate host cells towards preparing a niche for metastasizing tumor cells. Finally, TEX communicate with the matrix to support tumor cell motility, invasion and homing. We will discuss the possibility that CSC markers are the initial trigger for these processes and what is the special contribution of CSC-TEX.

Key words: Pancreatic cancer; Cancer stem cells; Stem cell markers; Exosomes; Crosstalk

Abstract

Pancreatic cancer (PaCa) has the highest death rate

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Core tip: Cancer progression relies on a small population of cancer stem cells (CSC), characterized by longevity, self renewal, drug resistance and requirement of a niche. In addition, CSC abundantly deliver exosomes (TEX) allowing CSC a long distance communication. At the descriptive level, CSC are characterized by a set of so called CSC markers. We here discuss for pancreatic cancer that the CSC markers CD44v6, c-Met, Tspan8, alpha6beta4, EpCAM, claudin7, CXCR4 and prominin1 can in a concerted activity account for all CSC features. This includes CSC TEX activity due to the engagement of CSC markers in TEX biogenesis and enrichment in TEX.

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INTRODUCTION

Pancreatic cancer (PaCa) has a dismal prognosis due to late diagnosis and early metastatic spread. Thus, there is an urgent need for improving diagnosis and for a better understanding of the mechanisms underlying PaCa progression. We briefly outline the state of the art in concern about diagnosis with emphasis on tumor exosomes (TEX) as a promising diagnostic tool and proceed to introduce cancer stem cells (CSC) including the processes of epithelial mesenchymal transition (EMT) and premetastatic niche formation. After introducing exosomes, we outline the functional activity of Pa-CSC markers and how they contribute to the dismal prognosis of PaCa.

Pancreatic cancer diagnosis

PaCa still holds the highest mortality rate, which is due to late diagnosis, early metastatic spread and drug and chemoresistance^[1]. Though the survival rate of patients with a tumor of < 1 cm is close to 100% and about 50% of patients with a tumor of < 2 cm survive, the 5-year survival rate for locally advanced PaCa is 9% and for metastatic PaCa 2%^[2], which is very demanding for approaching early diagnosis^[3-9].

Imaging advices (computed tomography, endoscopic ultrasound, emission tomography and combined computed tomography/positron emission tomography) are well established for therapy control. These new imaging advices have strongly improved PaCa detection, yet are still suboptimal for early detection^[3]. Therefore, imaging is frequently combined with additional serum biomarkers. The most common marker, carbohydrate-associated antigen 19-9 (CA19-9) is helpful in response monitoring and in taking a decision on resectability, but shows insufficient sensitivity and specificity

for early PaCa detection^[10]. Thus, the search for additional biomarkers is still ongoing^[11]. To name a few, mucin 1 (Muc-1), which is also detected in other malignancies, showed a minor improvement compared to CA19-9^[12]. It is suggested to be suited for early stage detection^[13]. DJ-1 (Parkinsonism associated deglycase) and combinations of regenerating family member 1 β , syncoilin, anterior gradient 2 with CA19-9 improve sensitivity and specificity^[14,15]. A serum proteome analysis of patients with PaCa showed significant upregulation of 40 proteins. Several of these proteins revealed disease associations to TP53^[16,17]. In addition, upregulation of galectin-1, gelsolin, lumican, 14-3-3 σ , cathepsin D, cofilin, moesin and plectin were described in PaCa patients. Gelsolin and lumican were suggested as markers to differentiate PaCa from chronic pancreatitis (CP)^[18-20]. The search for early serum PaCa markers also includes genetic and epigenetic markers^[21-23]. DNA methylation of basonuclin and ADAM metalloproteinase with thrombospondin type 1 motif 1 (ADAMTS1) in serum indicate prognostic valence^[24]. Recovery of hypermethylated TNFR superfamily member 10c, and apoptotic chromatin condensation inducer 1^[25] and of long noncoding (lnc) RNA metastasis associated lung adenocarcinoma transcript 1 are predictors of poor survival^[26]. Recovery of miR-21, miR-210, miR-155 and miR-196a in the serum allows differentiating PaCa patients from healthy donors. Recovery of these miRNA correlates with PaCa progression^[27]. When combining the evaluation of CA19-9, miR-155, miR-181a/b and miR-196a stage I PaCa could be detected and differentiated from CP^[28]. Serum miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185 and miR-191 allowed to differentiate PaCa from CP patients and healthy donors^[29]. A panel of 10 free serum miRNA indicated discrimination between tumor stages^[30]. A statistical meta-analysis confirmed free serum miRNA as a diagnostic tool in PaCa. However, none of these miRNA are selective for PaCa^[31]. Recently, TEX in serum, which allow concomitantly evaluating PaCa-promoted genetic, epigenetic, lipidomic and proteomic alterations^[23], received increased interest. A first report based on mutations in KRAS and TP53 revealed promising results^[32]. Another study reports on the recovery of miR-17-5p and miR-21 in serum TEX. Recovery of miR-17-5p and miR-21 in serum exosomes differs between PaCa and CP patients and correlates with tumor progression^[33]. The finding that glypican-1⁺ can be detected with 100% specificity and 100% sensitivity in serum TEX of PaCa patients attracted much attention. Notably, this included reliable detection of PanIN (pancreatic cancer *in situ*). Furthermore, the level of glypican-1⁺ TEX correlated with tumor burden and survival time. A mouse model with specific KRAS mutations promoting spontaneous PaCa development, confirmed recovery of glypican-1⁺ TEX at the stage of intraepithelial lesions^[34]. We were concerned about the recovery of Pa-CSC protein markers (CD44v6,

Tspan8, EpCAM, $\beta 4$ integrin) in serum TEX. Additionally, microarray screenings of PaCa serum and tumor line derived TEX suggested a panel of miR-1246, miR-4644, miR-3976, miR-4306 to be suited for PaCa diagnosis. Two findings should be mentioned. TEX-enclosed miRNA is recovered at a significantly higher level than free serum miRNA. Second, we recommend to evaluate both protein and miRNA markers, which improved sensitivity (100%) and specificity (80%)^[35].

These few studies on serum TEX require large scale controls. Yet, results so far appear promising for the long awaited early diagnosis of PaCa, where late diagnosis of PaCa becomes particularly vicious due to the early spread of PaCa^[36]. To shed light on the unexpected power of TEX, we introduce CSC including the process of epithelial mesenchymal transition (EMT) and the establishment of a premetastatic niche in advance of reasoning on the suggested linkage between Pa-CSC markers, TEX and tumor progression.

Cancer stem cells and the epithelial mesenchymal transition

The propensity to metastasize relies on the small subpopulation of CSC, named according to several joint features with embryonic and adult SC^[37]. CSC are long lived, can self renew and differentiate, slowly progress through the cell cycle, are radiation and drug resistant and account for primary tumor growth and metastatic spread^[38]. CSC and ESC share several signaling pathways, particularly overexpression of Oct4 (POU class5 homeobox1), Nanog (Nanog homeobox) and avian myelocytomatosis viral oncogene homolog (c-Myc)^[39] and signaling *via* Notch, Wnt and Hedgehog^[40], frequently initiating activation of the Ras-Raf-MAPK and PI3K-Akt pathway^[41].

The metastatic cascade of epithelial tumors is initiated through EMT^[42,43]. EMT essentially depends on CSC^[44,45]. The hallmarks of EMT are loss of cell-cell adhesion, *via* E-cadherin downregulation and gain in motility by remodeling of the cytoskeleton and formation of new cell-substrate contacts supported by intermediate filament proteins like vimentin^[43]. Initiation of the EMT program depends on a multitude of signals received from the environment that activate a corresponding array of intracellular signaling cascades^[46-48], which force expression of EMT transcription factors Twist, Snail, Slug, Zeb1 and others^[49]. Transforming growth factor (TGF) β is the major EMT inducer^[50], which signals through its receptors phosphorylating SMAD2 and SMAD3 that bind to SMAD4, the complex translocating to the nucleus^[50,51]. Wnt signals activate β -catenin that support Snail, but also vimentin transcription^[52-54]. Activation of the EMT program through receptor tyrosine kinase (RTK) ligands like HGF, EGF, FGF and PDGF (hepatocyte-, epidermal-, -fibroblast-, -platelet-derived growth factor), appears to be content dependent^[55-57].

EMT is initiated by downregulation of E-cadherin at the transcriptional and posttranscriptional level. EMT transcription factors are recruited to the E-cadherin promoter and repress transcription^[58]. Histone modifying enzymes cooperate in E-cadherin promoter repression. This includes polycomb group proteins, which form polycomb repressive complexes silencing transcription *via* modifying histones and recruiting additional repressors^[59]. Another important factor is Bmi1 that is upregulated in CSC and supposed to facilitate the EMT phenotype. Bmi1 downregulates Pten, which leads to activation of the PI3K/Akt pathway and posttranslational stabilization of Snail^[60]. Furthermore, Twist can bind to the Bmi1 promoter and upregulate its expression^[61]. Histone deacetylases are also engaged in E-cadherin silencing. They are either recruited by Snail^[62] or by Twist directly associated with the histone deacetylase complex^[63]. MiRNA presents the second major epigenetic mechanism engaged in the EMT process. In most instances miRNA binds to the untranslated region of their target genes, which prohibits target gene translation^[64]. The engagement of miRNA in EMT was first described for the miR-200 family. This family comprises miR-200a/b/c, miR-141 and miR-429. Decreased expression of the miR-200 family is accompanied by enhanced Zeb1 and Zeb2 expression^[65]. Additional miRNAs regulating EMT transcription factors are miR-29b, miR-30a, miR-205^[66-68]. Other EMT targets of miRNAs are E-cadherin (miR-9), N-cadherin (miR-194), Nestin and Star1 (miR-661), pulmonary adenoma resistance 3 (miR-491-5p), which is engaged in tight junction (TJ) distortion and p120 (catenin $\delta 1$) (miR-197)^[69-73]. Notably, some miRNA concomitantly regulate CSC and EMT. miR-200c becomes activated *via* p53, which binds to the miRNA promoter. As a consequence tumorigenicity and metastasis are suppressed^[74,75]. Also, by depletion of miR-21 the number of CSC decreases and EMT is reverted^[76]. In this context, it is important to remember that in epithelial cancer the process of EMT is transient^[77]. In line with this, the epithelial phenotype can be restored by a double-negative feedback loop, between Zeb, Snail1 and Gata3 and miR34a or miR-200^[78,79]. A similar feedback loop was described for miR-203 and Snail1^[80].

There is some debate, whether non-CSC by turning into the mesenchymal phenotype acquire CSC features or whether CSC transfer the required messages towards non-CSC^[44]. These options may not be mutually exclusive, taking the vision that CSC initiate the EMT phenotype in non-CSC, either by activating relevant signaling cascades by direct cell contact or *via* TEX, which could account for both binding initiated activation of signaling cascades and transfer of genetic and epigenetic information. The latter option has been most convincing demonstrated for the preparation of the premetastatic niche by CSC TEX.

Cancer stem cell niches and exosomes

CSC share with embryonic and adult SC dependence on a crosstalk with a special surrounding, called niche^[81,82]. Adult SC and CSC niches, which are important to maintain stemness, consist of epithelial and mesenchymal cells and extracellular substrates^[83]. An important contributor in the CSC niche are cancer-associated fibroblasts (CAF). CAF provide HGF, interleukin (IL)6, PDGF β , prostaglandins (PG) and proteases, which jointly remodel the extracellular matrix (ECM)^[84,85]. Other important players are mesenchymal stem cells (MSC)^[86], which cooperate with CAF and macrophages (M ϕ)^[87]. MSC are stimulated by tumor cell-derived IL1 to secrete PGE2, which operates in an autocrine manner promoting cytokine secretion and induces β -catenin signaling. These signaling cascades promote CSC conversion of adjacent non-CSC tumor cells^[88]. Stroma cell-derived tumor necrosis factor (TNF) α and IL6 sustain TGF β production and attract MSC to produce CSC supportive CXCL7^[89]. Tumor-derived growth factors stimulate resident fibroblasts to secrete fibronectin promoting CSC attachment. Stromal fibroblasts- and CAF-derived CXCL12 (stroma-derived factor 1, SDF1) attracts CXCR4 expressing hematopoietic, endothelial cell progenitors and CSC^[90]. c-Met becomes involved *via* HGF expressing MSC and β -catenin that together with the Tcf/Lef (lymphoid enhancer binding factor 1) complex translocates to the nucleus and initiate transcription of cell cycle related genes like *cyclin D1* and *c-Myc*^[91]. Activated integrin-linked kinase (ILK) further supports nuclear translocation of β -catenin, where ILK activation is promoted by matrix-bound β 1 integrins and costimulatory signals from the environment^[92]. Finally, there is evidence that niche maintenance is supported by a mutual exchange of miRNA between CIC and niche cells^[93-95]. Thus, SC actively recruit and activate those cells that in a feedback support their survival.

CSC also shape a niche for metastasizing tumor cells in selective organs in advance of tumor cell arrival, known as premetastatic niche. Tumor-derived growth factors stimulate resident fibroblasts to secrete fibronectin, which promotes attachment of hematopoietic progenitors expressing VEGF receptor (R)1 and α 4. In addition, stromal fibroblasts-derived CXCL12 attracts CXCR4 expressing hematopoietic progenitors and CSC^[96]. Meanwhile it is well established that TEX are the central actors in establishing a premetastatic niche in epithelial cancer^[97-99] including PaCa^[100,101].

Taken together, CSC maintenance depends on a crosstalk with the surrounding matrix and nearby as well as distant cells. There is strong evidence that TEX are the major player in this crosstalks.

Exosomes

Exosomes are small 40-100 nm vesicles. They are

delivered by many cells and abundantly by tumor cells^[102]. Exosomes biogenesis is initiated by the formation of early endosomes that become integrated as intraluminal vesicles (ILV) into multivesicular bodies (MVB). MVB can fuse with lysosomes for protein degradation. Alternatively, MVB fuse with the plasma membrane and release their ILV, which are termed exosomes^[103].

MVBs are assembled from early endosomes sorted from the trans-Golgi network or from internalized membranes, where the endosomal sorting complex required for transport (ESCRT) plays an important role in vesicle traffic and loading. The ESCRT complex is composed of the subcomplexes ESCRT I, II and III^[104]. *Tsg* (tumor susceptibility gene)101 in the ESCRT complex I binds ubiquitinated proteins and recruits ESCRT II. ESCRT II or Alix (ALG-2-interacting protein X) recruits ESCRT III. ESCRT III recruits a deubiquitinating enzyme that removes the ubiquitin tag from the cargo proteins prior to sorting into MVB^[105]. Finally, the ATPase vacuolar protein sorting 4 (Vsp4) dissociates the ESCRT III complex from the membrane. Additional essential partners in ESCRT-dependent exosome biogenesis are syndecans and transmembrane heparan sulfates, which interact with syntenin. Syntenin cooperates with CD63 and Alix^[106]. Alternatively, cell membrane integrated tetraspanins and other proteins residing in glycolipid-enriched microdomains (GEM)^[107] become incorporated into MVB, which is a sequel of the physical properties of GEM being prone for internalization^[108]. Indeed, tetraspanins are essential for exosome generation as demonstrated by defective exosome secretion in CD9 knockout mice^[109]. A third pathway proceeds *via* proteolipids (PLP). In cholesterol and ceramide-rich compartments, the PLP colocalize with flotillin and glycosylphosphatidylinositol. Exosome biogenesis *via* PLP depends on ceramide production by neutral sphingomyelinase-2. Sphingosine-1-phosphatase and diacylglycerol (DAG) are engaged in cargo sorting^[110].

Early endosomes hike through the cytoplasm in advance of being released as exosomes. Rab proteins, a subfamily of small GTPases, associate *via* geranylgeranyl modifications with membranes, regulate vesicle budding, tethering and fusion. Rab4 and rab5 mostly are recovered on early endosomes, rab11 is engaged in juxtannuclear recycling endosome traffic, rab7 and rab9 are recovered in late endosome and MVB. Rab35 and rab11 are engaged in endocytic recycling. Rab proteins regulate vesicle traffic *via* the interaction with actin and microtubules. Rab11 recruits myosin and dynein, moving of late endosomes along microtubules being dynein-dependent. Docking on the plasma membrane *via* kinesin is regulated by rab25. Rab GTPase activating proteins (GAP) and Rab27b are engaged in exosome release, where SNARE proteins (soluble-N-ethylmaleimide-sensitive fusion protein-attachment protein receptors) (v-SNARE) pair with

SNARE-binding partners (t-SNARE) on vesicles^[111]. Finally, during the invagination of early endosomes into MVB, the exosome cytoplasm receives its cargo (Figure 1A).

Exosomes are composed of a lipid bilayer containing transmembrane proteins. The small plasma contains proteins, mRNA, non-coding RNA and DNA. The potential cargo is estimated to approximately 100 proteins and 10000 nucleotides^[102]. The origin of the early endosomes determines the membrane lipid and protein composition of exosomes. Loading of the small plasma is a non-random, selective process that is not yet fully clarified.

As reviewed^[112], exosomes contain phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, prostaglandins, and lysobisphosphatidic acid and are enriched in sphingomyelin, cholesterol, GM3, and phosphatidylserine^[113]. Phosphatidic acid, diglycerides, and ceramides, lipid second messengers are involved in exosome biogenesis, where proteins of the ESCRT machinery interact with various lipids or lipid-related enzymes. Vps4 interacts with an oxysterol binding protein^[114] making a link with cholesterol metabolism. In fact, lipids in general, and more specifically sterols and fatty acids play a key role in Golgi/endosome/vacuole sorting^[115]. Furthermore, the high content of sphingomyelin, cholesterol and GM3 increase overall rigidity and stability^[116,117]. Phosphatidylserine facilitates exosome fusion and fission^[118] and lysobisphosphatidic acid is involved in intracellular fusion and budding^[119]. Packaging of miRNA into exosomes requires the neutral sphingomyelinase2^[120].

Exosomes contain approximately 7000 proteins^[121,122]. Constitutive exosomal proteins are structural vesicle components and proteins involved in vesicle biogenesis and trafficking. For GEM-derived exosomes, including tetraspanin networks, higher order oligomerization is important^[123]. There is strong evidence that exosomes derived from tetraspanin-enriched microdomains contain the unchanged membrane complex including attached cytoplasmic components^[124], which may account for GEM-derived exosomes in general. In raft-derived exosomes ceramide forming sphingolipids play an important role in exosome loading^[125]. Otherwise, mono-ubiquitination, acylation or myristoylation are known to facilitate sorting of proteins into exosomes^[126,127].

Tetraspanins are the most abundant exosome component^[107]. They are enriched 7-124 fold in exosomes compared to the parental cells^[128]. Additional abundantly recovered exosome components are adhesion molecules, proteases, MHC molecules, heat shock proteins (HSP), TSG101, Alix, annexins, cytoskeleton proteins (actins, cofilin-1, ezrin/radixin/moesin, profilin-1, tubulins), metabolic enzymes, cytosolic signal transduction molecules and ribosomal proteins. Some of these constitutive exosomal proteins are recruited *via* their association with proteins engaged in exosome biogenesis, which is well explored for

tetraspanin-associated integrins and proteases^[129,130], HSP-associated transferrin receptor and cytosolic proteins associated with transmembrane proteins or attached to the inner membrane of invagination prone GEM^[131]. Cell type-specific exosomal proteins are most comprehensively explored for cancer/CSC-TEX. Melanoma TEX contain MART1, epithelial cancer cell-derived TEX contain EpCAM and gastrointestinal cancer derived TEX contain cld7, glioblastoma TEX contain EGFRVIII and TEX of docetaxel-resistant prostate cancer cells contain multidrug resistance gene 1^[132-134]. TEX also contain c-Met, mutant KRAS and tissue factor^[132,135,136]. Notably, all CSC markers are recovered in TEX^[137,138].

Exosomes also contain mRNA, rRNA, tRNA, miRNA, lncRNA, mitochondrial DNA and short DNA sequences of retrotransposons^[139-141], protected from degradation by the double lipid membrane^[142,143]. RNA and DNA sorting into exosomes required further elaboration. Annexin-2 recruits specific RNAs by binding^[144]. A zip code in the 3'-UTR guides miRNA recruitment. It is facilitated by coupling of the RNA-induced silencing complex (RISC) to sorting complex components. GW182 containing GW bodies promote continuous assembly/disassembly of membrane-associated miRNA-loaded RISC. Finally, a specific EXO motif (GGAG) controls miRNA loading by binding to the heterogeneous ribonucleoprotein A2B1 (hnRNPA2B1), where sumoylated hnRNPA2B1 binds to an RNA transport signal (RTS or A2RE) in the 3' UTR containing the EXO motifs^[145]. The mechanisms for selective recruitment of lncRNA into exosomes remains to be explored^[146].

Though next-generation sequencing can be expected to shortly unravel exosomal DNA, coding and noncoding RNA^[147], microarray analysis already provided some valuable information, particularly on exosomal miRNA. MiRNA constitutes only 1%-3% of the human genome, but due to multiple targets, miRNA control about 30% of the coding genes. With perfect base pairing, mRNA is cleaved by Argonaut (AGO), upon imperfect binding, translation is repressed^[148]. Knowledge on miRNA greatly fostered progress in oncology. Selected miRNA could be linked to prognosis, disease progression, recurrence and metastasis^[149]. miRNA plays an important role in EMT^[150], maintenance of CSC^[151], tumor invasion, migration and angiogenesis^[152]. Most studies being not specifically concerned about TEX CSC miRNA, two publications should be mentioned that described selective TEX miRNA recovery in a subtype of CD44⁺ breast cancer cells^[153] as well as a report on CD133⁺ melanoma TEX that revealed 49 miRNA not detected in TEX from the parental cells, 20 of these selectively recruited miRNA displaying cancer related function^[154]. lncRNA makes up approximately 3% of the exosomal RNA. It also is transferred into host cells. Deep sequencing results are awaited for a profound evaluation on clinical relevance^[155] (Figure 1B).

Taken together, CSC/metastasizing tumor cells

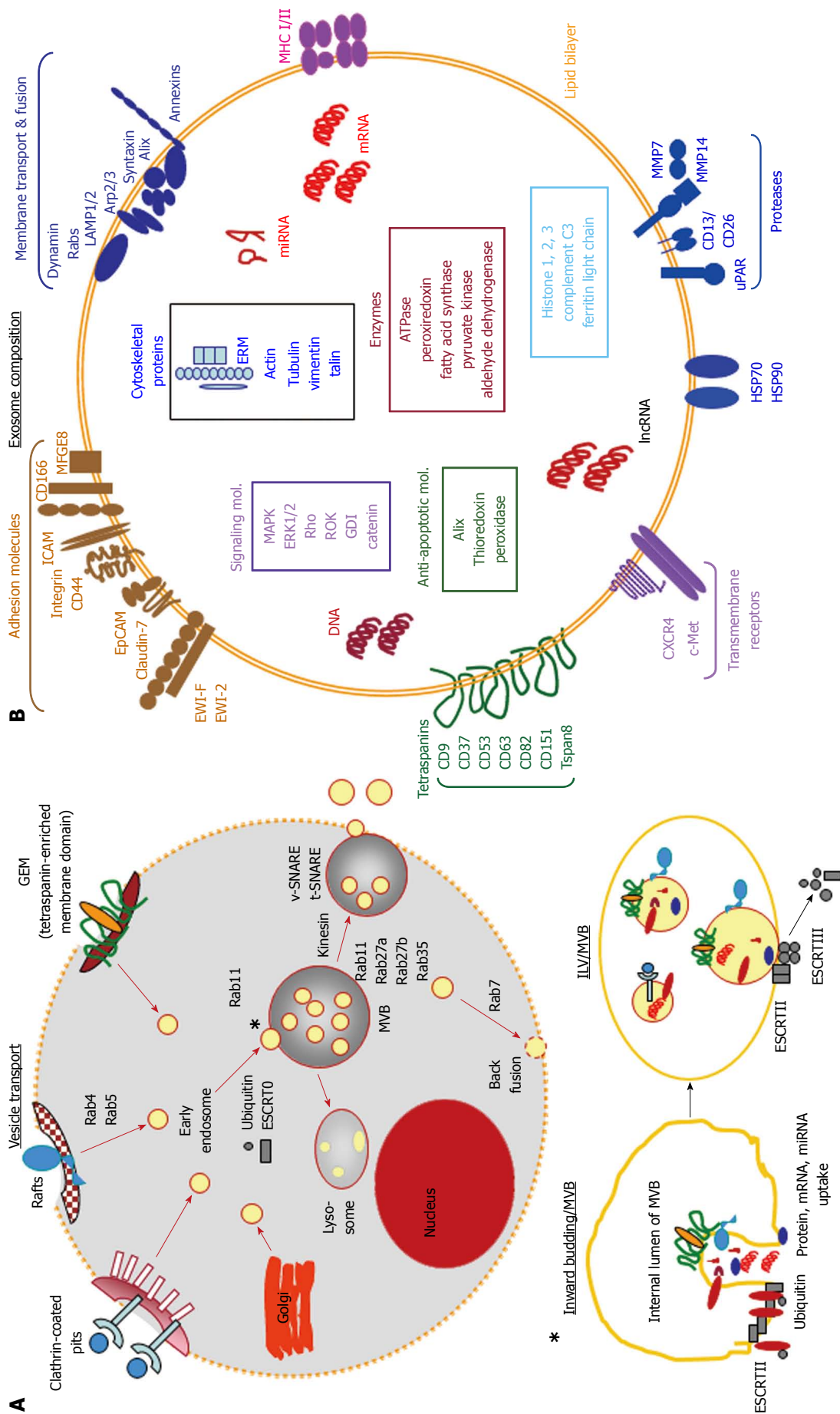


Figure 1 Exosome biogenesis and release. A: Exosome biogenesis is initiated by the generation of early endosomes delivered by the Golgi complex or by invagination of defined membrane microdomains such as clathrin coated pits, rafts and glycolipid enriched membrane microdomains, which are by scission separated from the originating membrane. Early endosomes are budding into multivesicular bodies. During this process the small cytoplasm of endosomes is loaded with proteins, mRNA and DNA. The invaginated early endosomes dissociate from the membrane of multivesicular bodies and are then called intraluminal vesicles. Early endosomes and multivesicular bodies are guided along microtubuli by transporter proteins and transporter complexes, where monoubiquitination, different Rabs, ESCRT complexes and SNARE proteins account for distinct guiding routes; B: Exosomes are characterized by a lipid bilayer enriched in cholesterol, sphingomyelin, GM3 and phosphatidylserine and membrane proteins that vary with the donor cell and the originating membrane microdomain, tetraspanins being a constitutive and highly enriched component. Signal transduction and cytoskeletal proteins are mostly recruited via the association with the inner plasma membrane. Cytosolic proteins, coding and noncoding RNA and DNA are selectively recruited. Of note, to our knowledge, all CSC markers are recovered and enriched in exosomes. CSC: Cancer stem cells; GEM: Glycolipid-enriched microdomains; ESCRT: Endosomal sorting complex required for transport; MVB: Multivesicular bodies; ILV: Intraluminal vesicles.

display SC features, which becomes most prominent during EMT and the establishment of and crosstalk with CSC niches including the premetastatic niche, supposed to be promoted by TEX. Thus, the question arose on the specific equipment of CSC that provides the base of these activities. Besides their functional characterization, CSC are defined by protein marker panels, which are frequently used for CSC/CSC-TEX isolation^[119-122]. Functional importance of these CSC markers only recently received attention. We hypothesize that CSC markers are the major players including the assembly of CSC-TEX.

PANCREATIC CANCER STEM CELLS MARKERS

Prominent Pa-CSC markers are CD44v6, c-Met, Tspan8, $\alpha 6\beta 4$, CXCR4, EpCAM and prominin-1 (CD133)^[35,156-162], most of which are also recovered in other gastrointestinal CSC. Importantly, these markers were demonstrated to be of functional relevance and to cooperate.

CD44v6 and c-MET

CD44v6 is a CSC marker in PaCa and colorectal adenocarcinoma (CoCa)^[35,162-166]. Its functional engagement was repeatedly demonstrated by the impact of CD44v6 overexpression and targeted deletion on metastasis formation^[100,167-169].

CD44v6 is a splice variant of CD44, an abundantly expressed adhesion molecule and the prime receptor for hyaluronan (HA)^[170], the globular N-terminal region also binds collagen, laminin, fibronectin (FN) and selectins^[171-173]. CD44v6 contains additional binding sites for the chemokine osteopontin (OPN)^[174,175], HGF and VEGF^[176,177] (Figure 2A). *Via* chemokine binding, CD44v6 becomes engaged in motility. OPN is chemotactic and haptotactic and as such important for cell recruitment^[178] and motility. Thus, p53^{ko}CD44^{ko} mice develop primary tumors at a comparable rate to p53^{ko} mice, but p53^{ko}CD44^{ko} tumors do not metastasize^[165,179]. CD44v6 has binding sites for cytokines. *Via* bound cytokines, CD44v6 takes over a coordinating role in RTK activation^[180,181], which is detailed below for the cooperation with c-Met. The cytoplasmic tail of CD44 plays an important role in signal transduction. It contains binding sites for the cytoskeletal proteins ezrin, radixin, moesin (ERM) and ankyrin. Ankyrin mediates contact with spectrin and is involved in adhesion and motility^[182]. ERM proteins are engaged in regulating migration, cell shape and protein resorting in the plasma membrane^[183]. The N-terminus of activated ERM proteins binds to CD44 and the C-terminus binds to F-actin, linking CD44 to the actin cytoskeleton^[184]. The binding of CD44 to cytoskeletal linker proteins influences signaling pathways downstream of CD44, which expands the range of CD44-mediated functions. Finally, CD44 can be cleaved by ADAMs and MMP-14^[185]. After

ectodomain cleavage, CD44 becomes accessible to the presenilin/ γ -secretase complex, which triggers intramembrane CD44 cleavage, setting free the CD44 intracellular domain (CD44-ICD). CD44-ICD acts as a co-transcription factor that potentiates beside others CD44, MMP9, MMP3 and HIF2 α transcription^[186-188]. The last point to mention is of central importance for the functional activity of CD44v6 as a CSC marker. CD44v6 O-glycosylation, the transmembrane region and the cytoplasmic tail affect the membrane subdomain localization, where recruitment into GEM^[189] promotes the interaction of CD44 with extracellular ligands and the association with other transmembrane and cytoplasmic molecules^[190]. These associations are most crucial for the activity of CD44 in signal transduction, migration, apoptosis resistance, premetastatic niche preparation^[191,192] and the cooperation with additional Pa-CSC markers^[98], one example being the recruitment of CXCR4 into GEM upon ligand binding, where it associates with CD44^[193,194].

Another CD44 feature of special importance for CSC migration is the cooperativity with proteases. CD44 concentrates MMPs at the cell surface and CD44 aggregation *via* HA binding further facilitates MMP binding^[187]. By the interaction with HA the production of uPAR, MMP2 and MMP9 is stimulated^[195]. Furthermore, the CD44-ICD binds to a MMP9 promoter response element actively supporting MMP9 transcription^[187]. ProMMP2 and proMMP9 become activated through CD44v-associated MMP14. Cell-bound MMPs being protected from their inhibitors, this allows for ECM degradation forming space for invading tumor cells^[196]. In addition, TGF β activation through CD44-associated MMP9, promotes angiogenesis and invasion and several mechanisms of TGF β -promoted apoptosis become silenced^[197,198] (Figure 2B-G).

Finally, as recently reviewed, CD44/CD44v6 and CD44/CD44v regulates miRNA engaged in metastasis^[180,199]. First to note, the CD44 3'-UTR binds several miRNA (miR-328, miR-491, miR-671, miR-512-3p) such that collagen 1 and FN are released from repression^[200]. Furthermore, upon activation Oct4-Sox2-Nanog are recruited to CD44v3 and translocate into the nucleus, where they initiate miR-302 transcription, which suppresses epigenetic regulators and increases expression of cIAP-1, cIAP-2 and XIAP strengthening drug resistance^[201]. We described abundant recovery of miR-494 and miR-542-3p in TEX from a CD44v6⁺ rat PaCa that promoted cadherin-17 downregulation accompanied by MMP release from repression^[202]. These sporadic findings will become consolidated by deep sequencing. Nonetheless, they provide first support for the engagement of CD44/CD44v6 in CSC activities also *via* miRNA.

Another Pa-CSC marker is the RTK c-Met^[156,203], which contribution relies at least in part on its cooperativity with CD44v6. c-Met becomes activated by binding its ligand HGF. As CD44v6 bind HGF, c-Met

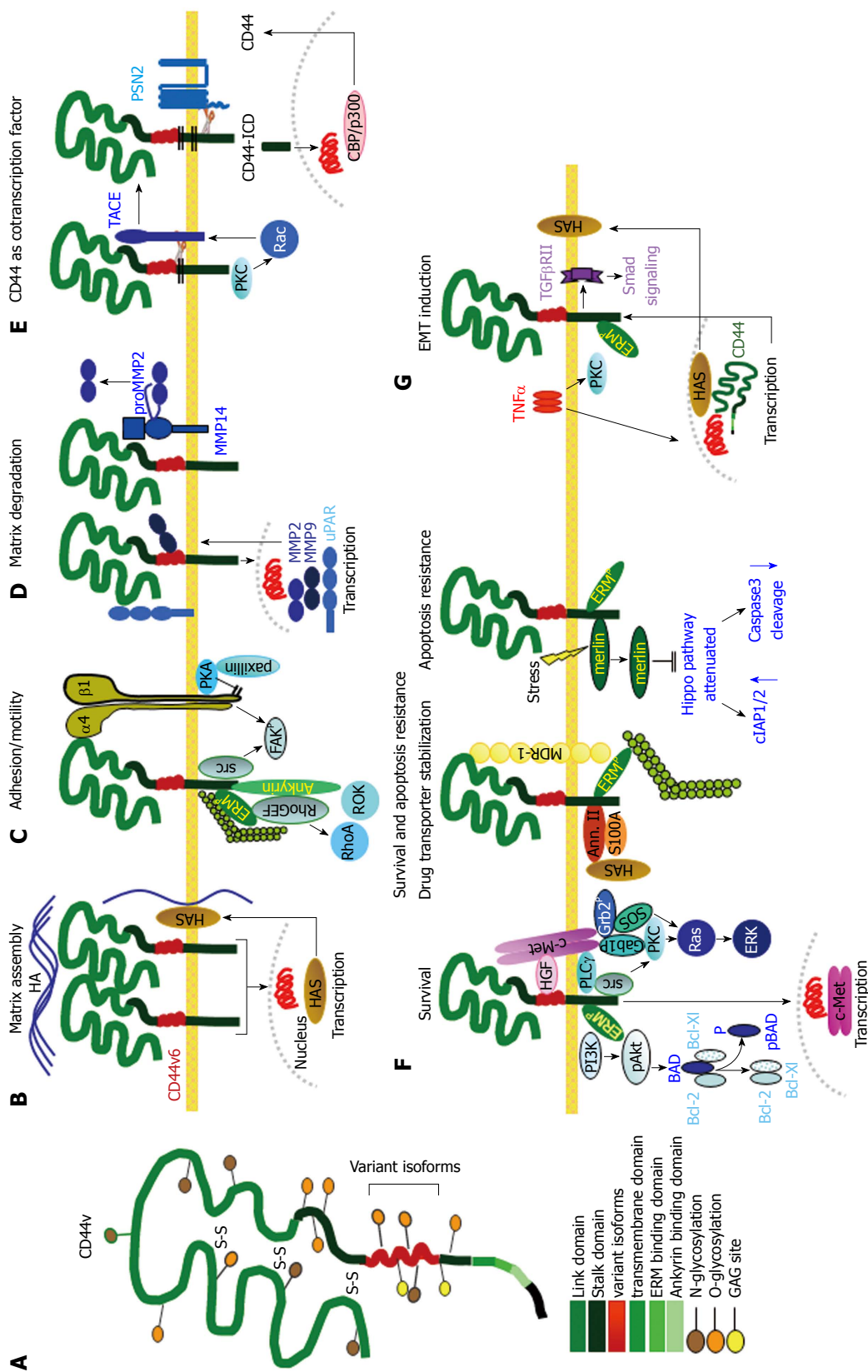


Figure 2 Cancer stem cells supporting activities of CD44v6. A: Structure of CD44 including insertion of variant exon products; B: CD44v6 is engaged in assembling the HA matrix by inducing HAS transcription. The matrix supports CD44 activation; C: GEM-located CD44/CD44v6 is src associated and cooperates with adjacent integrins. Activated ERM proteins and Ankyrin link CD44 to the cytoskeleton and initiate RhoA and ROK activation, which together play an important role in CSC motility; D: CD44v6 is engaged in uPAR and MMP transcription. It associates with MMP14, which captures MMP2 and MMP9, supporting ECM degradation and remodeling; E: GEM-located CD44v6 can be cleaved by TACE and subsequently by PSN2. CD44-ICD is a powerful cotranscription factor, which induces besides others CD44 transcription; F: There are several pathways, whereby CD44v6 supports CSC survival and apoptosis resistance. It cooperates with c-Met, which is brought into vicinity by CD44v6-bound HGF; a complex of CD44, Annexin II, S100A and HAS stabilizes MDR1; stress induces merlin phosphorylation, which dissociates from CD44 and hampers activation of the Hippo pathway; G: One pathway of CD44-linked induction of EMT relies on TNF α that fosters CD44 cooperativity with TGF β RI promoting Smad signaling. CD44 also is engaged in activation of Nanog and in repressing miRNA transcription that targets EMT proteins (not shown). The majority of these CSC supporting activities of CD44/CD44v6 rely on GEM-recruited CD44. CSC: Cancer stem cells; GEM: Glycolipid-enriched microdomains; ERM: Cytoskeletal proteins ezrin, radixin, moesin; EMT: Epithelial mesenchymal transition; TNF α : Tumor necrosis factor α ; TGF β : Transforming growth factor β ; TACE: TNF α converting enzyme.

comes into proximity of CD44v6, which contributes to c-Met activation. c-Met is a transmembrane heterodimer^[204]. Upon ligand binding the intracellular tyrosine kinase domain becomes activated through tyrosine phosphorylation in the carboxyterminal end providing docking sites for adaptor and intracellular kinases^[205]. The major adaptor protein is Grb2, prominent downstream signaling cascades are MAPK, PI3K/Akt and *via* these two pathways src, STAT3, nuclear factor κ B (NF κ B), FAK and β -catenin^[206]. CD44v6-initiated c-Met phosphorylation requires the cytoplasmic tail of CD44 and the interaction with ERM proteins for activation of the Ras-MAPK pathway^[204], the PI3K-Akt pathway and Wnt/ β -catenin signaling^[207,208]. In addition, CD44v6 regulates c-Met transcription^[100,209]. Similar observations account for the cooperation of CD44v6 with insulin-like growth factor-1- and PDGFR^[209,210]. Major cellular responses of c-Met activation include migration, invasion, stemness maintenance, apoptosis resistance and EMT. c-Met can directly interact with E-cadherin, which drives nuclear accumulation of β -catenin and leads to disruption of cell-cell adhesion^[211,212].

Tspan8 and $\alpha 6\beta 4$

Tetraspanins are a family of small proteins passing the membrane 4 times^[213]. Two family members, CD151 and Tspan8 are associated with tumor progression^[107,214,215]. For Tspan8 this accounts particularly for gastrointestinal cancer^[216-226], where we provided evidence that Tspan8 is enriched in Pa-CSC^[35,130,162]. What qualifies Tspan8 as a functionally relevant CSC marker?

Tetraspanin cross the membrane 4 times with the short N- and C-terminal tails being located in the cytoplasm. Tetraspanins have a small extracellular loop between transmembrane region 1 and 2 and a large extracellular loop between transmembrane regions 3 and 4. The large extracellular loop contains highly conserved cyteines that provide the essential signature of tetraspanins differentiating them from other 4-span molecules. The large extracellular loop accounts for dimerization and for interactions with non-tetraspanin partner molecules. Polar residues in the transmembrane regions stabilize the tertiary structures^[227-230]. Palmitoylation is required for initiating tetraspanin-tetraspanin web formation, protects from degradation and provides a link to cholesterol and gangliosides, which supports the formation of GEM^[231-236]. Some tetraspanins avail on a tyrosine-based sorting motif that promotes internalization. Yet, internalization can also proceed *via* associated molecules with a sorting motif^[237-239] (Figure 3A).

With few exceptions, tetraspanins have no direct ligands. Instead, they form complexes by interacting between themselves and a large variety of transmembrane and cytosolic proteins^[240]. The most prominent tetraspanin partners are integrins^[241,242],

for Tspan8 particularly $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ ^[243-245], but $\alpha 4\beta 1$ and $\alpha 5\beta 1$ also associate with Tspan8^[246,247]. Proteases are an additional class of functionally important tetraspanin partners^[248], Tspan8 associating with the dipeptidase CD26, MMP14, TACE (ADAM17), MMP2 and 9^[130,226,245,246,249]. Tetraspanins associate with growth factor receptors^[250,251], G protein coupled receptors (GPCR) and their intracellular associated heterotrimeric G-proteins^[252] as demonstrated for the relaxin receptor in prostate cancer^[253]. Prominent cytosolic signal transduction molecules co-immunoprecipitating with tetraspanins are protein kinase C (PKC), a type II phosphatidylinositol 4 kinase (PI4KII) and phospholipase $C\gamma$ (PLC γ)^[254-256], these associations being also relevant for Tspan8^[243,257]. Most important for the activity of Tspan8 as Pa-CSC marker are the associations with $\alpha 6\beta 4$, CD44v6 and EpCAM^[243-245,258,259].

Besides providing a signaling platform, tetraspanin complex location in GEM facilitate vesicular fusion and/or fission^[107,260-262], which is supported by a tyrosine-based sorting motif of tetraspanins or associated proteins^[263].

Taking into account the reversibility of palmitoylation and the instability of membrane microdomains, it can be expected that tetraspanin activities vary considerably depending on the activation state of the cell. The fact that tetraspanins act *via* laterally associated molecules and only exceptionally *via* ligand binding, promotes their large array of functions. Nonetheless, there is a common theme. Tetraspanins promote adhesion, spreading, motility, cable formation, invasion, membrane microdomain internalization and vesicle formation. These activities rely on integrin compartmentalization, internalization, modulation of integrin signaling and integrin biosynthesis^[241,242,264]. Invasiveness depends on the association with proteases or could proceed through modulating MMP transcription and secretion^[245,248]. The involvement of tetraspanins in fusion events has been convincingly demonstrated by the failure of egg-sperm fusion in CD9 and CD81 knockout mice^[265], the involvement in cell-virus and cell-parasite interactions^[266,267] and their morphogenic features^[268,269].

Tspan8 shares most of these activities with other tetraspanins^[107]. In gastric cancer Tspan8 promotes metastasis *via* activation of the MAPK pathway^[223]. It contributes in particular *via* its strong association with $\alpha 6\beta 4$, which is only seen upon $\alpha 6\beta 4$ activation by ligand binding. The Tspan8- $\alpha 6\beta 4$ association strikingly increases tumor cell motility and is accompanied by ezrin, paxillin, src, FAK, and rac/ras activation^[245]. Dysregulated adhesion and motility also account for colorectal cancer metastasis^[216]. Invasion is supported by the association with TACE, MMP2 and MMP9 and a weak association with MMP14, which could be indirect *via* the association with CD44v6^[245]. In esophageal cancer, too, cooperativity between

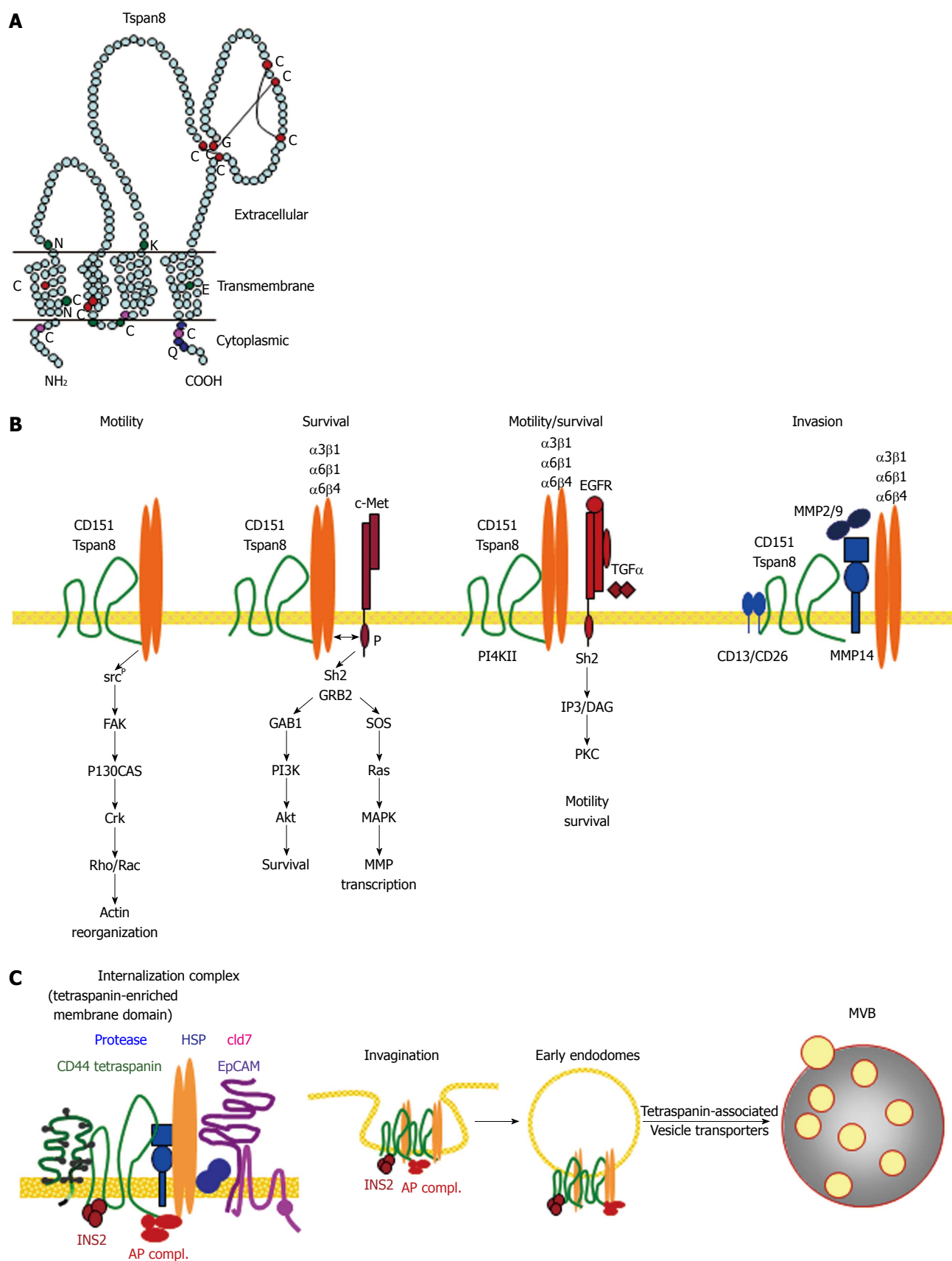


Figure 3 Cancer stem cells supporting activities of Tspan8. A: The structure of Tspan8 showing the prominent large extracellular loop, which is the main binding site for laterally associated molecules; B: Dominant partners of Tspan8 and CD151 are integrins and proteases. Tspan8 and CD151 also associate with RTK, CD44v6 and EpCAM. The integrin associations promote CSC motility, the RTK associations survival and the protease associations invasiveness; C: the strongest contribution of Tspan8 in support of CSC relies on its engagement in GEM complex located molecule internalization. Tspan8 is also involved in vesicle traffic. The importance of Tspan8 in CSC is linked to recruiting additional CSC markers into GEM and in contributing to the transfer of the GEM complex into TEX. CSC: Cancer stem cells; GEM: Glycolipid-enriched microdomains; MVB: Multivesicular bodies; PKC: Phosphokinase C; RTK: Receptor tyrosine kinase.

Tspan8 and ADAM12m promotes metastases^[226]. We consider the engagement in EMT gene transcription *via* its association with β -catenin^[130] and Notch^[270], also described for CD44^[271] and EpCAM/cld7-associated EpCAM^[272,273] and the cooperativity with CD44v6, $\alpha 6\beta 4$ and the EpCAM-cld7 complex^[98,266] as most important for the contribution of Tspan8 to the CSC phenotype of PaCa (Figure 3B). The contribution of Tspan8 to exosome generation (Figure 3C) and, as outlined below to targeting^[107], adds to the central importance of Tspan8 in Pa-CSC.

As mentioned, one of the Tspan8 partners is the $\alpha 6\beta 4$ integrin, the linkage between $\alpha 6\beta 4$ and the tetraspanins CD151 and Tspan8 being repeatedly reported^[264,274,275] and ample evidence is provided for the engagement of $\alpha 6\beta 4$ in PaCa progression^[130,243,276-280].

The $\alpha 6\beta 4$ integrin is unique in structure and subcellular localization. Distinct to other β chains, the cytoplasmic domain of $\beta 4$ is over 1000 amino acids long. Towards the C terminus it contains two pairs of type III fibronectin-like modules, which contain tyrosine phosphorylation and proteolytic cleavage sites. Furthermore in the resting state $\alpha 6\beta 4$ is located in hemidesmosomes anchoring epithelial cells *via* laminin binding to the basement membrane, indicating its interaction with keratin filaments opposing the actin filament association of other integrin β chains^[281]. However, upon stimulation, *e.g.*, by wounding, stress and in tumor cells, hemidesmosomes become disassembled and $\alpha 6\beta 4$ is driven into GEM, preferentially in F-actin protrusions^[207,264,282-284]. Palmitoylation of the $\beta 4$ chain support the GEM localization and $\beta 4$ initiated signal transduction^[285,286]. Upon disassembly of hemidesmosomes, the $\beta 4$ cytoplasmic domain becomes phosphorylated preferentially *via* PKC α ^[287,288]. Phosphorylated $\alpha 6\beta 4$ binding to laminin activates both PI3K and ras homolog family member A (RhoA) small GTPases^[280,289,290]. Alternatively to laminin binding, $\alpha 6\beta 4$ activation can be initiated by cooperation with growth factor receptors including ErbB-1,2,3 and c-Met^[207,290-296], which promotes activation of PI3K, Akt, MAPK, and Rho small GTPases pathways^[207,289,297-299].

$\alpha 6\beta 4$ affects cell survival and angiogenesis^[244,289,297,300,301] and was reported to alter expression of > 500 genes^[302]. Its dominating activity relies in promoting tumor cell invasiveness^[130,274,278,279,290], which fits well to its association with tetraspanins in GEM. Notably, there is evidence for engagement in stemness^[35,130,162,303-306]. In PaCa it is predominantly associated with Tspan8 and expression is upregulated in Pa-CSC^[35,162]. The impact of this association may gain further weight by the joint recovery in PaCa TEX^[130,204].

CXCR4

CXCR4 is a G protein-coupled chemokine receptor^[307], upregulated in CSC, particularly migrating CSC^[308] including metastatic PaCa and lung cancer cells with CSC-like properties, which show upregulated CXCR4 and CD133 expression^[309,310]. CXCR4 is suggested to

contribute to tumor growth, angiogenesis, therapy resistance^[90,311-313] and to have a strong impact on metastasis including the recruitment to specific sites such as the bone marrow^[314]. In 85% of PaCa CXCR4 expression is increased and was identified as an independent factor for poor prognosis^[162,315,316].

After stromal-derived factor (SDF)1 binding CXCR4 and possibly extracellular HSP90 colocalize to lipid rafts, which facilitate together with HSP90 signal transduction^[317]. Activated CXCR4 increases intracellular calcium levels and induces a phosphorylation cascade, which is terminated by CXCR4 internalization^[318]. After chemokine binding the heterotrimeric G protein is activated and dissociates in GTP-bound α and $\beta\gamma$ subunits. Cell motility is regulated by several phosphorylation cascades, which include src and Akt as the central node. Akt phosphorylates several downstream targets that reorganize actin fibers. The $\beta\gamma$ subunit activates PLC β and PI3K. PLC β cleaves PIP2 in IP3 and DAG, where IP3 induces the release of Ca from intracellular stores. DAG together with Ca activates PKC and MAPK. PI3K activation leads to activation of focal adhesion components and cytoskeletal proteins contributing to reorganization of the actin cytoskeleton. Actin polymerization is stabilized by HSP90, which promotes the formation of filopodia and directed cell migration^[319]. Activated PI3K additionally activates *via* Akt the mitochondrial antiapoptotic signaling pathway^[320]. Activated Akt also contributes to β -catenin stabilization and gene transcription^[321]. Signaling through Gai is linked to transcription through PI3K/Akt, NF κ B, mitogen-activated protein kinase kinase 1/2 and leads to activation of the Ras and Rac/Rho pathways^[322]. Ligand binding induced dimerization results in G-protein-independent signaling with activation of the JAK/Stat pathway^[323], which might be accompanied by polarization^[324]. Finally, CD44 binding to human epidermal growth factor receptor 2 (HER2) supports CXCR4 expression in gastric cancer by suppressing transcription of miR-139, which targets CXCR4^[325].

There are several reports on the association of CXCR4 with tetraspanins in hematological malignancies^[326,327], where the GEM located complex cointernalizes and is recovered in TEX^[328]. We recovered CXCR4 and Tspan8 in PaCa TEX^[35,130] and CXCR4 expressing TEX from a metastatic CoCa line promote metastasis formation of poorly metastatic lines. The authors speculate that this is due to recruiting CXCR4⁺ stroma cells to create a metastasis-permissive environment^[329].

In brief, CXCR4 increases the motility of metastasizing CSC. Recruitment into GEM facilitates cooperativity with addition GEM-located CSC markers as well as internalization and recovery in TEX (Figure 4).

EpCAM and claudin 7

EpCAM (EpC) is a prominent CSC-marker in colorectal, pancreatic, liver and breast cancer^[330-332], but infor-

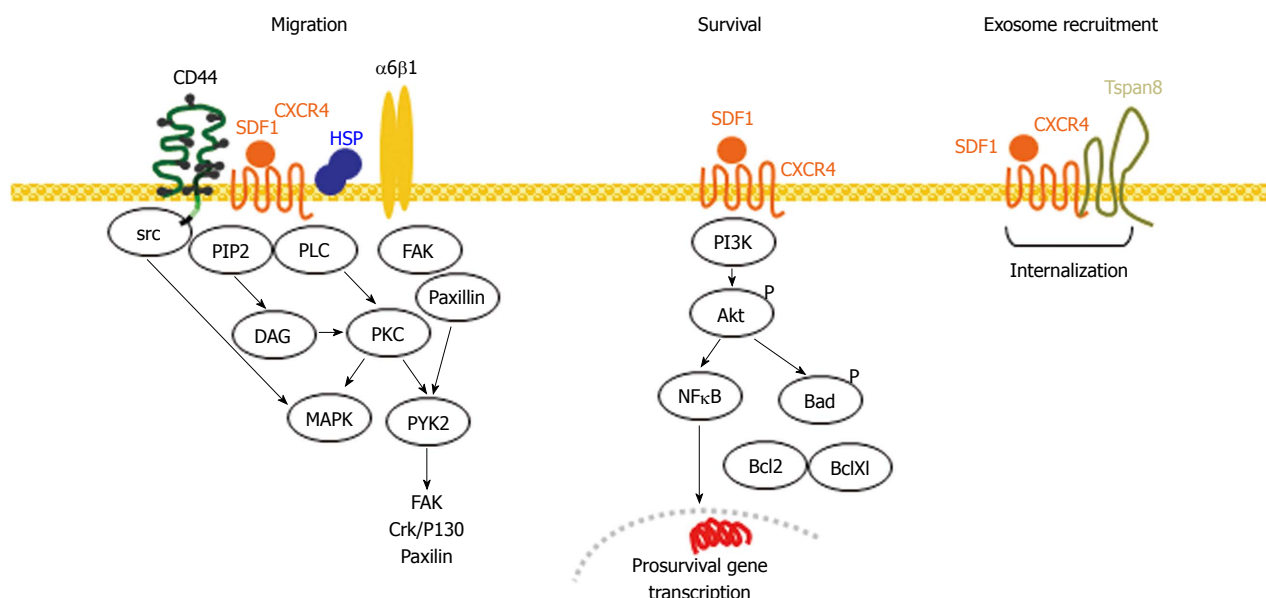


Figure 4 CXCR4, a marker of migrating cancer stem cells. CXCR4 becomes activated by SDF1 binding, which in concert with CD44, integrins and HSP initiates several signaling cascades that promote directed motility. CXCR4 also activates the PI3K/Akt pathway promoting antiapoptotic protein activation and pro-survival gene transcription. Activated CXCR4 becomes recruited into GEM, where in Pa-CSC the association with Tspan8 supports internalization. CXCR4 is recovered in TEX. In Pa-CSC CXCR4 cooperates with CD44(v6), laminin binding integrins and Tspan8 in TEM and is recruited into TEX. CSC: Cancer stem cells; GEM: Glycolipid-enriched microdomains; NF- κ B: Nuclear factor κ B; TEX: Tumor exosomes; HSP: Heat shock protein; PLC: Phospholipase C; PKC: Phosphokinase C; PI3K: Phosphatidylinositol-4,5-bisphosphate 3 kinase; MAPK: Mitogen-activated protein kinase; SDF1: Stroma-derived factor 1.

mation is limited, whether EpC fulfills CSC-related tasks^[333-336]. In gastrointestinal cancer evidence was provided that CSC activity of EpCAM requires support by claudin 7 (cld7)^[259,273,337-341].

EpC is a tetramer forming transmembrane molecules, which mediates homophilic cell-cell adhesion^[342]. This weak homophilic binding is only seen in E-Cadherin cells due to EpC interfering with E-cadherin *via* disrupting the link between β -catenin and F-actin^[343]. Due to a response element to the Wnt downstream effector Tcf4 it fosters Wnt signaling responses^[52,344]. However, EpC can also act as a Wnt derepressor *via* sustaining Lrp6 (LDL receptor related protein 6) retention^[345]. EpC also can control motility *via* down-regulation of PKC^[346] and by regulating MMP7 expression^[347,348]. These activities are promoted by the cytoplasmic tail of EpCAM (EpICD), which forms a complex with β -catenin, FHL2 (four-and-half-LIM-only) and Lef-1. The complex relocates to the nucleus, initiating, c-myc, cyclinA and E transcription^[349]. The finding that EpC cross-linking triggers TACE (TNF α converting enzyme), which cuts the extracellular domain such that the membrane-anchored intracellular domain becomes accessible to PSN2 (presenilin 2 N-terminal fragment), which cleaves the intracellular peptide, EpICD, opened a new window towards the activity of EpC as a CSC marker^[11]. EpICD also initiates transcription of additional reprogramming genes like Oct4 and Nanog, which is accompanied by EMT with upregulation of vimentin, Snail, Slug and downregulation of E-cadherin in a murine colon cancer and a human hepatoma line^[350]. This study did not

take into account the expression of cld7. However, hepatocyte progenitors express, besides EpC, cld7^[351] and in CoCa and PaCa, EpC associates with cld7^[235]. Under physiological conditions, too, the EpC-cld7 association is vital, an EpC^{ko} mouse dying within one week after birth due to intestine destruction, which relies on the missing association of EpC with cld7^[352]. These findings pointed towards a concerted activity of EpC and cld7 in tumor progression, which was confirmed by a cld7^{kd} and an EpC^{kd} in a metastasizing line. Both knockdowns sufficed to wave metastatic growth^[341].

Claudins, four-pass transmembrane proteins, were first described as TJ components that are engaged in sealing, formation of ion channels and organization of paracellular small organic solute flux^[353-355]. The importance of clds, including cld7, was repeatedly demonstrated by targeted deletion. A cld7^{ko} is lethal within 10 d after birth due to intestine destruction^[356]. The authors speculate that gut destruction is promoted by a missing association with integrins and upregulation of MMPs. An intestine-specific conditional cld7^{ko} mouse revealed a specific enhancement of paracellular small organic solute flux across the TJ including a major bacterial product that initiates colonic inflammation^[357].

However, claudins are also found outside of TJ^[358-362]. Claudins are PKA, PKC and myosin light chain kinase targets^[363-367]. Importantly, cld phosphorylation can prohibit integration into tight junctions with the consequence of loss of epithelial cell polarization^[368-370]. Cld7 also has palmitoylation sites^[36,356,371] and palmitoylation

toylated cld7 is excluded from TJ^[371], but partitioned into GEM, where it is associated with monomeric EpCAM^[273,341,372]. As already mentioned, GEM harbor palmitoylated proteins and act as a scaffold for signal transduction and reorganization of the cytoskeleton^[373-376]. GEM-located, palmitoylated cld7 promotes tumor progression by supporting motility and invasion. This was confirmed in PaCa and CoCa for the EpC-cld7 complex, which promotes motility and invasion^[259,341,372] as well as drug resistance that is initiated by downregulation of Pten^[341]. There is additional evidence for a shift towards EMT gene expression^[273], palmitoylated cld7 contributing to the generation of EpICD^[371], facilitated by the GEM location of TACE and PSN2. Further supporting the cld7-EpC complex functioning as a CSC marker, triple negative breast cancer cells are cld7⁺, but cld7-associated rab25 is expressed in breast-CSC^[377-379]. Finally, outlined above, GEM are prone for internalization and recruitment into exosomes, which facilitate the metastatic process^[380], where we experienced that cld7 actively contributes to the vesicle transport *via* associating with vesicle transporters. In CoCa and Pa-CSC the EpC-cld7 complex is recovered in TEX^[273,361].

Taken together, cld7 and palmitoylated cld7 apparently account for distinct, non-overlapping activities such that dependent on the cellular context, the functional engagement in TJ or in GEM is dominating. Only GEM-located, palmitoylated cld7 displays CSC activity, where EpC contributes due to its association with GEM-located cld7. The main activity of this CSC marker complex builds on apoptosis resistance and EMT gene expression (Figure 5). A contribution of cld7 to TEX biogenesis might further strengthen the impact on CSC activity.

Prominin-1

Prominin-1 (CD133) is a CSC marker in several cancer entities^[381-384], including PaCa^[156,308,385-390]. CD133 is a pentaspan protein^[391,392]. It is suggested to be associated with the Notch pathway, which is accompanied by slow cell cycling and increased drug resistance^[393,394] as well as Hedgehog signaling with an increased capacity of anchorage independent growth^[395]. CD133 is also engaged in Akt, JNK, mTOR, MAPK and IL-8/CXCL1 signaling cascades^[396]. These findings are well in line with CD133 supporting maintenance of stemness and point towards a possible engagement in EMT gene transcription. Furthermore, it is well documented that CD133 interacts with cholesterol and is concentrated in different types of membrane protrusions with different types of cytoskeletal bases, *i.e.*, actin for microvilli and tubulin for cilia. These different membrane protrusions also appear to be released in at least two types of vesicles^[396,397]. The smaller vesicles resembles exosomes containing all the constitutive exosomal proteins and the exosomal lipid profile. These

exosomes, which also contain prometastatic proteins like CD44 and ADAMs, are taken up by tumor cells and bone marrow derived stroma cells, the transfer of CD133 being accompanied by increased invasiveness and metastatic potential^[154]. Whether the second type of exosomes proceeds *via* the PLP pathway^[110], which could be suggested by the interaction of CD133 with cholesterol, remains to be elaborated. Irrespective of their origin, CD133⁺ TEX are recovered in CoCa and PaCa^[35,159,361].

Taken together, CD133 is engaged in multiple signaling pathways linked to metastasis and EMT. CD133 also is another Pa-CSC marker that is constitutively located in internalization-prone membrane domains and is recovered in TEX.

In brief, the dominating features of the Pa-CSC markers are their connectivity, their engagement in multiple signaling pathways and their location in internalization prone membrane domains, which accounts for the enriched recovery in TEX. By these characteristics, Pa-CSC markers are prone to contribute to motility, invasiveness and EMT. *Via* their enrichment in TEX they appear destined for the crosstalk with the host and non-CSC.

PANCREATIC CANCER STEM CELL MARKERS AND THE EPITHELIAL MESENCHYMAL TRANSITION

There are different modes, whereby Pa-CSC markers can contribute to EMT, markers can be engaged in the regulation of EMT gene transcription factors, EMT gene related miRNA processing or can be targets of miRNA. Last, not least, they can be engaged in the transfer of EMT transcription factors or miRNA into TEX, where TEX could become the actual transporter of EMT. So far information on these topics are rather limited. One hindrance being the transient nature of EMT, which becomes aggravated by the definition of CSC as a population of cells, enriched but not purified by a variety of distinct procedures. An additional hindrance relies on the evaluation of overall expression of CSC markers, which does not take into account that CSC markers are mostly recruited into GEM, where they can fulfill distinct or opposing functions compared to activities outside of GEM, like anchoring epithelial cells to the lamina basalis ($\alpha 6 \beta 4$) or contributing to epithelial cell sealing (cld7). Nonetheless, there are reports describing regulation of CSC markers by EMT genes and vice versa.

Pancreatic cancer stem cell markers and EMT gene regulation

There are several reports on the engagement of CD44 in EMT gene regulation. In PaCa Snail-1 is a downstream target of CD44. Snail regulates MMP14 expression, which supports invasion^[398]. Another

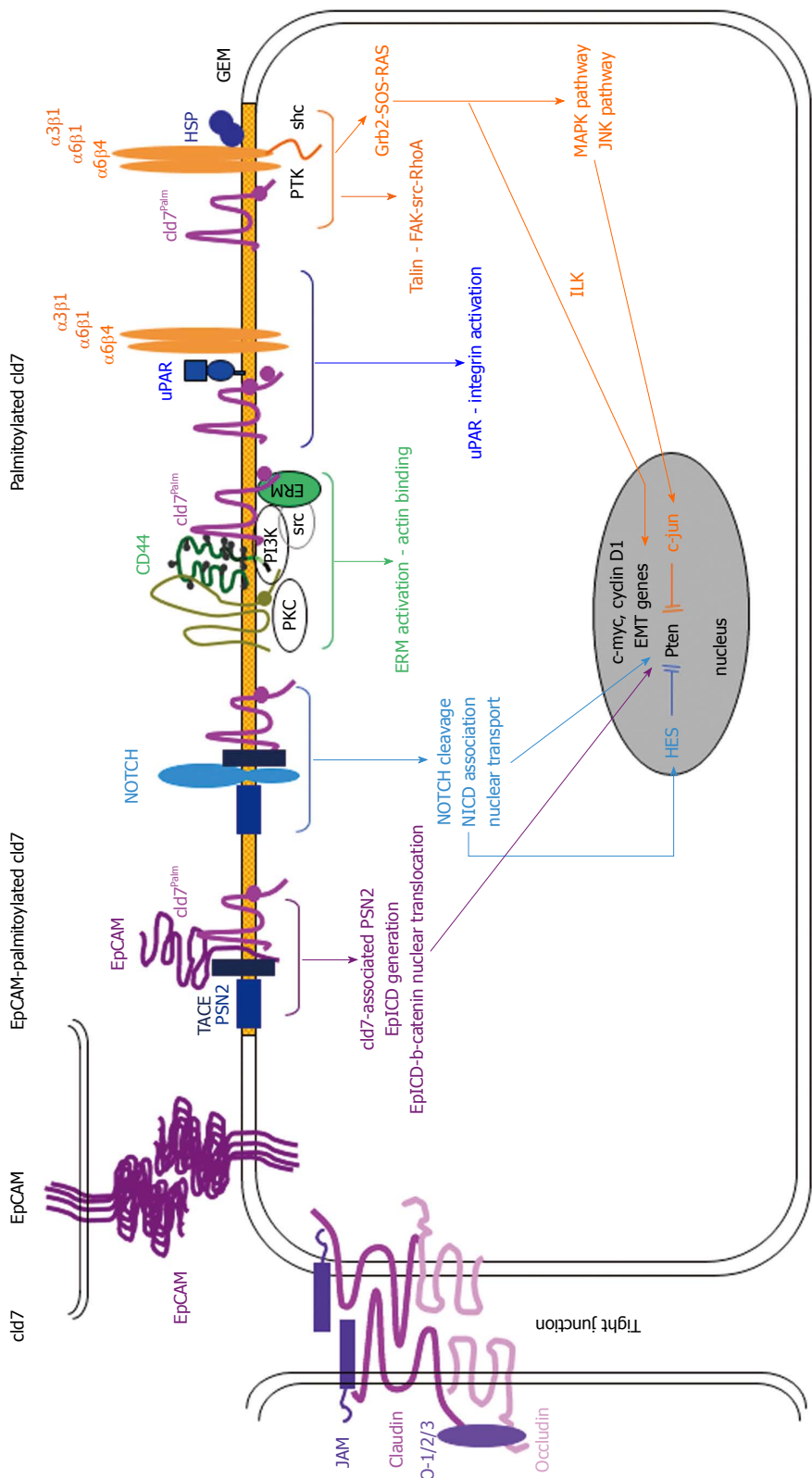


Figure 5 The EpCAM-palmitoylated claudin7 complex in pancreatic cancer stem cells. Upon palmitoylation the TJ protein claudin7 becomes excluded from TJ and recruited into GEM, where it associates with monomeric EpC. Monomeric EpC becomes susceptible to GEM-located TACE and PSN2. EpICD associates with β -catenin and translocates to the nucleus acting as a cotranscription factor for c-myc, cyclinD1 and several EMT genes. The cld7-TACE-PSN2 complex also contributes to NOTCH cleavage. NICD contributes to HES activation, which inhibits Pten transcription. The palmitoylated cld7-uPAR association promotes integrin activation and transcription of EMT genes via ILK. Activation of the JNK pathway contributes to inhibition of Pten transcription. In Pa-CSC EpC becomes cleaved and involved in EMT gene transcription; palmitoylated cld7 additionally is engaged via Notch cleavage and integrin activation in apoptosis resistance. CSC: Cancer stem cells; GEM: Glycolipid-enriched microdomains; ILK: Integrin-linked kinase; TJ: Tight junction; HSP: Heat shock protein; PKC: Phosphokinase C; TACE: TNF α converting enzyme.

EMT transcription factor linked to CD44 is Zeb1. There is a self reinforcing feedback loop as Zeb1 and CD44 mutually sustain their expression. Notably, this study also describes an inverse linkage to CD44v expression, which is due to Zeb1 suppressing transcription of the epithelial splicing regulatory protein 1 (ESRP1)^[39]. An excess of HA production also drives EMT, accompanied by upregulation of TGF β and induction of Snail and Twist. Accordingly, inhibition of TGF β -Snail signaling or Twist silencing abrogated the entrance into a stem cell state^[40]. Furthermore, STAT3 is physically linked to CD44 and NF κ B. This initiates the activation the catalytic subunit of telomerase (hTERT), which functions as a transcription cofactor in EMT^[41]. Overexpression of Notch-1 induces CD44 and EpC expression and increases the formation of PaCa sphere formation, accompanied by the induction of the EMT markers Zeb1 and Hes-1^[402]. In thyroid Ca, CD44-ICD binds to CREB, which promotes

cyclinD1 transcription^[403] and in hepatocellular CA, a CD44/TM4SF5 (tetraspanin L six family member 5) association leads to activation of src, STAT3, Twist1 and Bmi1, supporting establishing the CSC phenotype and EMT^[404]. In CoCa the CD44-HA ligation initiates src activation, which supports Snail activation that represses the stemness inhibitor miR-203^[405]. Finally, GEM-located CD44 becomes internalized and migrates together with acetylated STAT3 to the nucleus. Nuclear CD44 binds to the promoters of several genes including c-myc and Twist1, promoting the EMT shift^[406].

The tetraspanin TM4SF5 also is involved in EMT induction. TGF β 1-mediated Smad activation causes TM4SF5 expression, EMT and EGFR pathway activation. The finding that inhibition of EGFR activity abolished EMT suggests a link between Smad and the EGFR in TM4SF5 expression. In fact, inhibition of Smad or the epidermal growth factor receptor (EGFR) blocked TM4SF5 expression and EMT^[407]. In human hepatocellular carcinoma cell lines TM4SF5 expression correlates with enhanced p27Kip1 (cyclin-dependent kinase inhibitor 1B) expression and cytosolic stabilization. Cells acquire an elongated phenotype, which relates to RhoA inactivation and loss of E-cadherin expression is accompanied by EMT^[408]. In glioma, KITENIN (VANGL planar cell polarity protein 1), a tetraspanin partner, induces expression of the EMT markers N-cadherin, Zeb1, Zeb2, Snail and Slug and expression of the CSC markers CD133, aldehyde dehydrogenase 1 and ephrin receptor B1^[409]. Signaling through TIMP-1 (metallopeptidase inhibitor 1) induces in breast cancer in dependence of CD63 Twist1 expression, where a knockdown of Twist1 rescues E-cadherin expression^[410]. In PaCa, upregulation of Notch-1 depends on Tspan8, similar effects being not induced by CD151^[130]. Instead, in mammary progenitor cells CD151 accounts for nuclear distribution of Slug and represses mammary branching morphogenesis^[306], whereas in ovarian cancer the CD151- α 3 β 1 complex represses Slug-mediated EMT and Wnt signaling^[411]. Similar, highest level of CD63 in melanoma revealed a significant resistance to undergo an EMT program^[412].

The CSC marker CXCR4, too, was described to contribute to EMT. Constitutively active CXCR4, but not wild type CXCR4 induces EMT in mammary carcinoma cells, characterized by upregulation of Zeb1, upregulation of cadherin 11, p120 isoform switching, activation of ERK1/2 and MMP2, but loss of E-cadherin. In 3-dimensional cultures, wt CXCR4 also suffices promoting EMT, which is accompanied by CXCR2, CXCR7, CXCL1, CXCL8, CCL2, IL6 and GM-CSF expression. Inhibition of CXCR4 together with MAPK1 or PI3K reversed the EMT phenotype^[413]. UHRF1 (ubiquitin-like, with PHD and RING finger domains 1) plays a crucial role in DNA CpG methylation, chromatin remodeling and gene expression. Downregulation of UHRF1 induces Zeb1 and Snail expression accom-

panied by decreased E-cadherin and increased N-cadherin and vimentin expression. The authors speculate that activation of the CXCR4 signaling pathway is of central importance^[414].

EpC is well accepted as a CSC marker, but reports on its contribution to EMT are opposing. One study with breast cancer cells reports on the contribution of EpC in TGF β 1-induced EMT. TGF β 1 treatment induced EpC expression, which promoted EMT and cell migration. EpC overexpression further enhanced TGF β 1-induced EMT. TGF β 1 treatment induces JNK phosphorylation that promoted increased Jun and Fos expression suggesting an important role of EpC in the induction of EMT *via* JNK signaling^[415]. Opposing findings were reported for prostate cancer, where EpC was repressed upon induction of EMT. miR-200c and miR-205 are two inducers of MET (mesenchymal-epithelial transition). Re-induction of the epithelial phenotype through miR-200c and miR-205 was accompanied by EpC reexpression^[416]. Instead, we reported on unaltered EpC and increased cld7 expression in PaCa and CoCa spheres/holo-clones and migrating tumor cells^[341]. Recruitment of monomeric EpC into GEM *via* palmitoylated cld7 and EpC cleavage could well account for EpC initiating pronounced EMT induction^[273,371]. Furthermore, we and other groups reported on upregulation of GEM-located palmitoylated cld7 in CSC and a pronounced release of the EpC-cld7 complex into TEX^[273,361], which promote Snail, Slug and Twist expression^[273]. Opposing findings have also been reported, where a knockdown of cld7 induced EMT. A cld7 signature gene profile revealed highly upregulated Rab25, a CoCa suppressor and regulator of polarized cell trafficking in cld7 overexpressing cells. Rab25 silencing counteracted the effects of cld7 expression and increased p-src and Erk1/2 expression^[417]. The study did not take into account the engagement of rab25 in vesicle traffic. Further elaborating the recruitment of the EpC-cld7 complex into GEM and exosomes may clarify these seemingly opposing findings.

Finally, CD133 overexpression induces "stemness" properties in PaCa cells and EMT. EMT induction and increased invasiveness are mediated by NF κ B activation^[418].

Thus, CD44v6, c-Met, Tspan8, α 6 β 4, CXCR4 and CD133 are engaged in promoting EMT. We and others provided evidence for a contribution of an EpC-cld7 complex in EMT. However, this topic is still controversial.

Pancreatic cancer stem cell markers, EMT and miRNA

There is abundant information on altered miRNA profiles in cancer, including CSC and tumor cells in EMT. For more detailed information on miRNA in PaCa excellent reviews are available, besides others in^[137,419-422]. Thus, we will mention only a few publications referring explicitly to the mutual impact of CSC markers on miRNA and vice versa.

HA-activated CD44 binds Twist, which supports transcription of miR-10b, which blocks the tumor

suppressor HOXD10 allowing for RhoA and ROK activation with consequences on organization of the cytoskeleton/tumor cell motility as well as apoptosis resistance *via* activation of the PI3K/Akt pathway. Activated CD44 also binds to Nanog, which together with Stat3 translocates to the nucleus and initiates miR-21 transcription, which downregulates the tumor suppressor PDCD4 and promotes expression of survival proteins^[423]. HA-activated CD44v3 interacts with Oct4, Sox2 and Nanog, stimulating miR-302 expression, which leads to downregulation of epigenetic regulators and activation of survival proteins^[424]. CD44-bound HER2 induces histone deacetylation accounting for transcriptional repression of miR-139, which targets CXCR4, the finding providing a link between upregulated expression of CD44 and CXCR4 in gastrointestinal CSC^[325]. Notch-1-induced increased miR-21 and decreased miR-200b, miR-200c, let-7a, let-7b and let-7c expression is accompanied by upregulation of the CSC surface markers CD44 and EpC^[402]. Up-regulated miR-155 significantly increases the population of CSCs as well as EMT in liver cancer cells *via* silencing TP53INP1 (tumor protein p53 inducible nuclear protein 1), changes being initiated by TGF β 1 that indirectly regulates TP53INP1 *via* induction of miR-155^[425]. miR-34a induces MET *via* down-regulation of Snail by binding to the Snail 3'-UTR, which is accompanied by down-regulation of Bmi1, CD44, CD133, olfactomedin and c-myc. Conversely, Snail and Zeb1 bind to E-boxes in the miR-34a/b/c promoters, which represses miR-34a and miR-34b/c expression. Thus, inactivation of miR-34a/b/c, which is frequent in cancer, can shift the equilibrium of these reciprocal regulations towards EMT^[78]. Sonic hedgehog signaling also becomes engaged in EMT by downregulation of miR-200b and let-7c with concomitant upregulation of CSC markers^[426]. In CoCa, miR-142-3p targets CD133, Lgr5 (leucine-rich repeat containing G protein-coupled receptor 5) and ABCG2, where Oct4 suppresses miR-142-3, expression being particularly low in CSC-enriched spheres^[427]. In PaCa miR-34 is lost in the population of CSC, which is accompanied by Notch and Bcl2 pathway activation, transcription of miR-34 being regulated by p53^[428]. However, it should be mentioned that most of these studies were oriented towards therapy and evaluated in first instance the regulation of EMT transcription factors, their reduction expectedly correlating with CSC marker expression, which excludes in several instances a statement on a direct impact of these miRNA on CSC marker expression. A miRNA analysis of rat and human PaCa with downregulation of the CSC markers CD44v6, EpC, cld7 and Tspan8^[35,202] confirmed low level miR-34a recovery in CD44v6-competent PaCa and upregulated expression in CD44v6^{kd} PaCa, which is in line with miR-34a targeting CD44^[429]. Furthermore, miR-103 transcription is more than

two-fold increased in TEX from CD44v6-competent compared to CD44v6-deficient cells. As c-Met supports miR-103 transcription^[430], the finding indicates that *via* CD44v6 c-Met also becomes engaged in miRNA transcription and/or posttranscriptional regulation. Finally, CD44v6-related changes are mostly reflected in the TEX miRNA profile such that miRNA reduced in CD44v6^{kd} cells is also lower in TEX from CD44v6^{kd} than CD44v6-competent cells^[202]. Tspan8 and cld7 exerted a pronounced effect mostly on miRNA known to be engaged in EMT gene expression. The functional relevance remains to be explored.

In brief, there is evidence for an impact of CSC markers on miRNA expression/repression. miRNA also affects CSC marker expression directly or *via* EMT genes and involved signaling pathways. Still, we are far from having a precise overview of these interlinked networks.

CONTRIBUTION OF PANCREATIC CANCER STEM CELL MARKERS TO TEX

Pancreatic cancer stem cell markers and recruitment of proteins and miRNA into TEX

We already outlined the engagement of Pa-CSC markers in TEX biogenesis, where GEM located Tspan8 plays a decisive role in early endosome formation^[124,129]. CD44v6, α 6 β 4, the EpC-cld7 complex and partly CD133 are co-recruited due to enrichment in these tetraspanin-dominated microdomains. According to unpublished findings, palmitoylated cld7 is actively engaged in early endosome traffic towards MVB and the release of ILV as exosomes. Fittingly, Tspan8, α 6 β 4, CD44v6, cld7 and CD133 are enriched in TEX compared to Pa-CSC^[162]. Comparative analyses of miRNA in TEX derived from Pa-CSC marker-expressing vs -depleted cells confirmed enriched recovery of EMT-related miRNA in Pa-CSC TEX and indicated an additional loss, respectively, enrichment of several miRNA related to the metastatic process, which still requires elaboration of the routing into TEX^[202]. Similar findings were reported at the proteome level by the group of Rak. TEX from A431 cells that were driven into EMT exhibit profound qualitative differences in their proteome compared to TEX from the parental cells, but also differed from the A431-EMT cells with 30 proteins related to growth, signaling and motility being uniquely recruited into A431-EMT-derived TEX. The authors propose that changes in the cellular differentiation status translate into unique qualitative rearrangements in the cargo of TEX^[431]. Along this line, the oncoprotein latent membrane protein 1 (LMP1) recruits HIF1 α into TEX of nasopharyngeal carcinoma. TEX HIF1 α remains function-competent in recipient cells, LMP1⁺ and HIF1 α ⁺ TEX initiating EMT with reverting the expression of E- and N-cadherins in TEX target cells^[432].

Contribution of pancreatic cancer stem cell markers to TEX binding and uptake

The power of exosomes relies on their ubiquitous presence, their particular protein, mRNA, ncRNA and DNA profile and their most efficient binding and/or transfer in target cells. Information on the latter aspect, though a prerequisite for clinical translation, is still limited.

Binding of PaCa TEX to the extracellular matrix (ECM) varies with the adhesion molecule profile of the exosomes. Thus, high CD44 expression is accompanied by HA binding and high $\alpha 6 \beta 4$ expression by laminin (LN) 332 binding, the findings being confirmed by antibody blocking^[433]. Myeloma cell line- and myeloma patient-derived TEX revealed fibronectin as key heparan sulfate-binding ligand and mediator of TEX-cell interactions, where removal of heparan sulfate from TEX dramatically inhibiting TEX-target cell interactions. The authors describe a dual role of heparan sulfate in TEX-cell interaction. TEX heparan sulfate captures FN. Concomitantly it acts as a FN receptor on target cells^[434]. Live-cell imaging also revealed a critical role of FN and integrin cargo sorting into TEX, which promoted persisting cell motility^[435]. In line with the latter report, there is abundant evidence for the engagement of integrins in exosome binding. During reticulocyte maturation, integrin $\alpha 4 \beta 1$ is recruited into exosomes, which bind to FN. The interaction depends on divalent cations and is inhibited by an $\alpha 4$ -specific antibody, the authors speculating on functional activity of exosomal $\alpha 4 \beta 1$ by binding to endothelial cells through CD54^[436]. B cell exosomes also interact with the ECM and fibroblasts *via* $\beta 1$ and $\beta 2$ integrins, antibody blocking studies confirming engagement in adhesion to collagen-I and FN and to activated fibroblasts *via* TNF α ^[437]. TEX of a PaCa transiently interfered with leukocyte migration. This is due to TEX occupying the migration-promoting receptors CD44, $\alpha 4$, CD62L and CD54^[438]. T cells, too, recruit dendritic cell (DC) exosomes not *via* the T cell receptor complex, but *via* leukocyte function-associated antigen-1 (LFA1)^[439]. Most impressive has been the elucidation that TEX integrin profiles account for the organ preference of metastasis initiated by formation of a premetastatic niche. A proteome analysis revealed distinct integrin expression patterns in subpopulations of TEX. Notably, exosomal integrins $\alpha 6 \beta 4$ and $\alpha 6 \beta 1$ were associated with lung metastasis, whereas exosomal integrin $\alpha v \beta 5$ was linked to liver metastasis. A blockade of $\alpha 6 \beta 4$ or $\alpha v \beta 5$ decreased TEX uptake, as well as lung, respectively, liver metastasis. Furthermore, TEX from mouse and human tumors are preferentially taken up by resident cells at their predicted metastatic destination, *i.e.*, TEX of tumors metastasizing to the lung are taken up by lung fibroblasts and epithelial cells, TEX of tumors that metastasize to the liver are captured by Kupffer cells and TEX from tumors metastasizing to the brain

are recovered in brain endothelial cells (EC). Finally, TEX integrins displayed functional activity, activating Src phosphorylation and pro-inflammatory S100 gene expression after uptake by resident cells^[440]. These studies confirmed and expanded our previous work that described distinct TEX integrins to target *e.g.*, EC, fibroblasts or bone marrow cells, where the selectivity of TEX uptake is guided or, at least, facilitated by the engagement of protein complexes at the exosome and the target cell membranes^[441]. In fact, only defined tetraspanin-integrin complexes are taken up by selected target cells. Importantly, TEX uptake proceeds *via* binding to internalization prone microdomains^[129]. The constitutively high expression of GEM-located tetraspanins and the multitude of associated molecules, with a preference for integrins^[107], favors our suggestion. Besides supporting the selectivity of binding and uptake, the engagement of complexes of TEX receptors and target cell ligands favors induction of signal transduction as *e.g.*, known for T cell activation, which requires engagement of the T cell receptor and accessory molecules that interact with MHC and costimulatory molecules on DC^[442]. So far we supported our hypothesis by elaborating that TEX expressing Tspan8 and $\alpha 4 \beta 1$ preferentially target EC and promote EC and EC progenitor activation^[247], where exosomes from Tspan8 and $\alpha 4$ transfected fibroblasts exhibited a comparable target cell selectivity^[129]. Instead, PaCa TEX expressing Tspan8 and $\alpha 6 \beta 4$ preferentially bind and are taken up by lymph node stroma cells and lung fibroblasts^[130], lymph nodes and lung being the exclusive metastatic sites for this PaCa^[443].

Taken together, for GEM-derived TEX there is strong evidence for preferential uptake by corresponding, internalization prone membrane microdomains. Furthermore, the work by the Lyden group depicting TEX integrins accounting for metastasis organ preference^[440] and our work on the engagement of tetraspanin-integrin complexes facilitating selective targeting^[129,130,247,443] provides a solid base for defining GEM-derived TEX target structures. Comparable studies for TEX uptake *via* phosphatidylserine receptors, by phagocytosis, macropinocytosis and membrane fusion^[444-449] are still awaited (Figure 6A).

TEX, PANCREATIC CANCER STEM CELL MARKERS AND THE CROSSTALK WITH THE HOST

In advance of reviewing the impact of Pa-CSC markers on angiogenesis and the premetastatic niche, we want to refer to excellent reviews that elaborate the impact of TEX on tumorigenicity^[98,450], tumor growth related thrombosis^[451-453], hematopoiesis^[199,454,455] and mature leukocytes, including all components of the immune system^[456-463], where particularly the Pa-CSC TEX

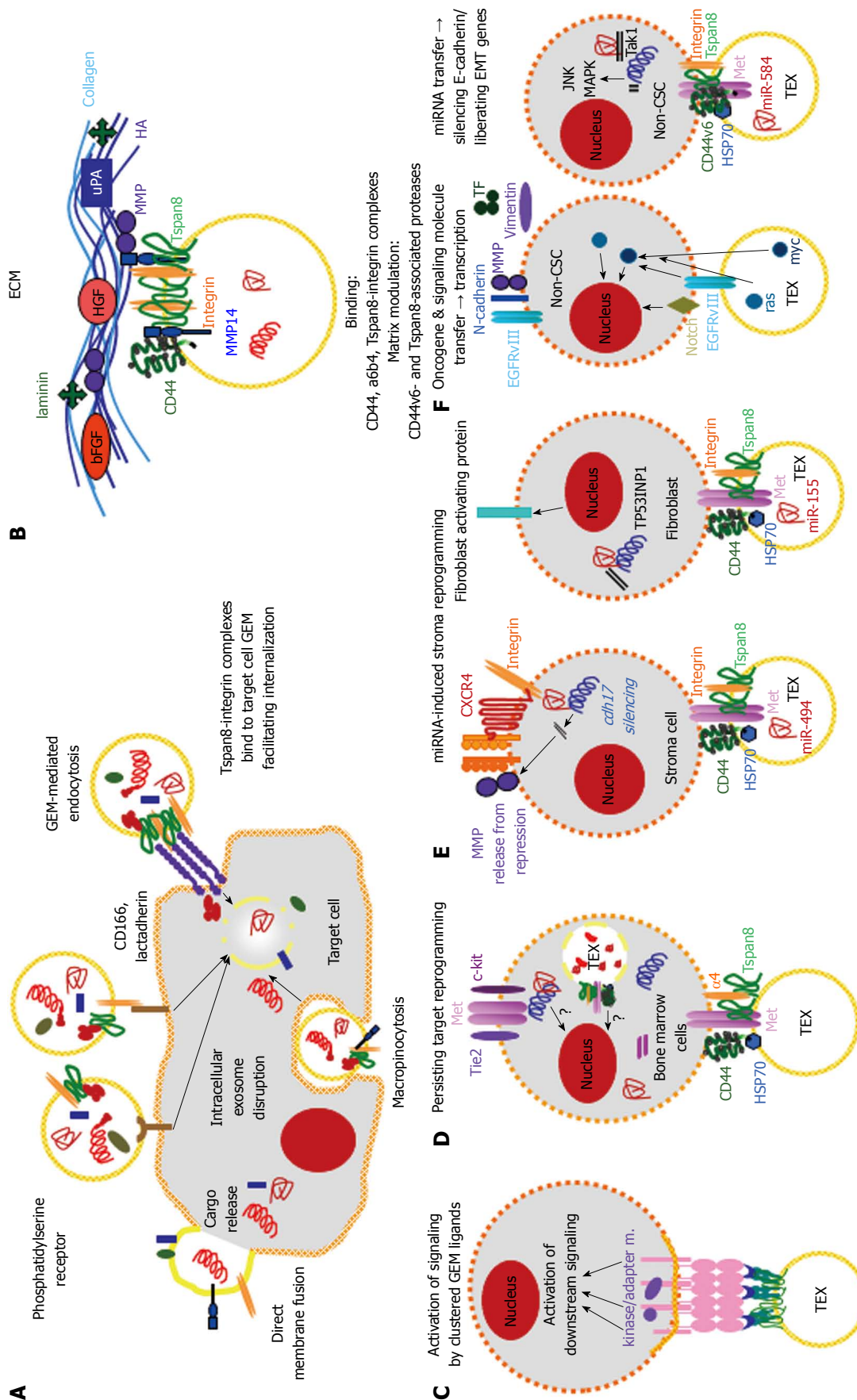


Figure 6 Contribution of exosomal cancer stem cell markers to target selection, binding, uptake and target modulation. A: Exosomes uptake by cells can proceed via membrane fusion, macropinocytosis, receptor ligand binding. GEM-derived exosomes bind to complexes of ligands located in internalization prone membrane microdomains, which increases selectivity of uptake and facilitates uptake; B: Binding to the ECM is facilitated by the Pa-CSC markers $\alpha\beta4$ and CD44v6. CD44v6- and Tspan8-associated proteases facilitate matrix degradation; C-E: There are multiple pathways whereby TEX affect target cells, only selected examples are shown: Signal transduction can be initiated by clustering GEM ligands; in progenitor cells, e.g., in the bone marrow, activation of signaling molecules can initiate a shift towards differentiation; the transfer of TEX miRNA can initiate release from repression by the miRNA target or tumor suppressor RNA can become silenced such that resting mesenchymal cells turn into an activated phenotype; F: EMT was induced in Non-CSC by the transfer of oncogenes, activation of EMT-related transcription factors or miRNA blocking transcription of RNA engaged in epithelial stage maintenance. CSC: Cancer stem cells; GEM: Glycolipid-enriched microdomains; ECM: Extracellular matrix; EMT: Epithelial mesenchymal transition; HA: Hyaluronan; TEX: Tumor exosomes.

markers CXCR4, CD44v6 and c-Met may play a role.

Contribution of pancreatic cancer stem cell TEX markers to angiogenesis

There is ample evidence on the engagement of TEX in angiogenesis. It was first described for TEX of a non-metastatic rat pancreatic cancer that induced overshooting angiogenesis resulting in a lethal consumption coagulopathy^[464]. TEX were preferentially taken up by EC and EC progenitors, binding and uptake requiring a Tspan8- α 4 β 1 complex. Notably, by exchange of α 4 β 1 by α 6 β 4, TEX did not bind to EC and overshooting angiogenesis was prevented^[246]. Uptake of Tspan8- α 4 β 1 TEX by EC resulted in upregulation of tissue factor, VEGFR1, CXCL5, CCR1 and HMOX1 as well as of Tpan8 and CD31. Depending on the culture condition, progenitor cells could also be driven into smooth muscle cell differentiation^[250]. We are not aware on further studies on Pa-CSC TEX markers in angiogenesis. Therefore, and as TEX-induced angiogenesis meanwhile is described in nearly all tumor entities, we refer to some reviews on TEX-initiated signal transduction in EC as well as on the engagement of transferred miRNA^[152,465-467]. However, we want to mention that to our knowledge, the first report on exosomes induced angiogenesis referred to platelet-derived exosomes^[468], which we interpret as an additional evidence that CSC take over physiological programs including the use of exosomes.

Pancreatic cancer stem cell markers, TEX and the crosstalk with the host

Paving the way for metastasizing tumor cells:

Exosomes are rich in function-competent proteases. Exosome proteases can modulate the exosome protein profile, the ECM and/or target cells. Besides others, MMP2, 7, 9, 14, ADAM10, 15, 17, ADAMTS1, 13 and several dipeptidases were recovered in TEX^[469,470]. These TEX proteases can modulate the TEX protein profile, which includes the Pa-CSC markers CD44, shedded by ADAM10, MMP14 and MMP9^[471-473], and EpC, shedded by ADAM14^[474]. Besides this internal regulation of CD44v6 and EpC expression in TEX, frequently accompanied by release of the ICD, which in turn promotes transcription of genes promoting tumorigenicity and metastasis^[187,349,350], the association of TEX CD44v6 and Tspan8 with proteases severely affects the host matrix. The modulated matrix, in turn, facilitates metastasizing tumor cell migration towards the metastatic organ.

HA is the most abundant ECM protein, with TEX binding *via* CD44^[170,475]. Notably, TEX also contain HAS and Hyal and were described to be HA-coated. The authors speculate that TEX serve as special vehicle for HA, where exosomal HA itself or associated molecules could create an environment supporting cancer cell invasion and metastasis^[476]. In concern about the contribution of CD44v6, we noted in some, but not all

tested TEX of PaCa-CD44v6^{kd} lines a reduction in HAS3 and upregulated expression of Hyal1. In addition, CD44v6-competent, but not CD44v6-deficient TEX-modulated HA promotes tumor cell migration^[100].

CD44 regulates expression and cooperates with several proteases^[195,196,477], where MMP2, MMP3, MMP7, MMP9, MMP14 as well as ADAM10 and ADAM17 are recovered in PaCa TEX and MMP9, MMP14 and ADAM17 are strongly downregulated in TEX of CD44v6^{kd} lines^[433]. These proteases coimmunoprecipitate with CD44v6 in PaCa-TEX, indicating their recruitment into TEX *via* associated CD44v6^[433]. CD44v6-competent PaCa TEX degrade coll I, coll IV, FN, LN111 and, less pronounced LN332, matrix degradation by PaCa TEX being accompanied by pronounced tumor cell migration and invasion^[433]. Similar findings were reported for MMP14, where the authors suggest that coll IV, which is not a MMP14 target, becomes degraded by MMP14-activated proMMP-2^[470]. Finally, host matrix degradation by CD44v6-competent TEX is accompanied by activation of proliferation and survival signals^[433]. This is likely due to liberation of growth factors, chemokines and additional proteases from the degraded matrix as well as by cleavage of additional targets by the TEX proteases^[207,478].

Tspan8 also associates with proteases, particularly MMP9 and TACE^[245] and Tspan8-associated proteases are recovered in PaCa TEX, where they degrade the host matrix^[130]. The efficacy of Tspan8-expressing TEX appears to exceed that of CD44v6⁺ TEX, which likely is due to the strong association of tetraspanins with integrins^[241,242]. Thereby matrix protein binding becomes focalized, strengthening the efficacy of matrix degradation. This accounts in particular for LN332 degradation. Due to its association with TACE, Tspan8 also contributes to FN degradation^[130]. Though we focused on the contribution of the Pa-CSC marker Tspan8 in matrix modulation, other TEX tetraspanin-protease complexes also contribute to host matrix modulation^[130,460,479-484].

In brief (Figure 6B), TEX proteases modulate the ECM thereby creating a path for migrating PaCa cells and a milieu favoring tumor cell migration, angiogenesis and premetastatic niche establishment. The Pa-CSC markers CD44v6 and Tspan8 essentially contribute to the process of matrix modulation by TEX due to their engagement in protease transcription (CD44v6), TEX biogenesis (Tspan8) and their association with proteases in GEM (CD44v6 and Tspan8).

Preparing a niche: TEX uptake remodels recipient non-tumor cells towards driving tumor growth. After the first description of a premetastatic niche^[96], the engagement of TEX soon became obvious, which we were the first to describe for a rat PaCa-CD44v6^{kd} line that had lost the capacity of the parental line to metastasize, but regained metastatic capacity, when rats were pretreated with TEX of the parental

line^[100]. Similarly, renal CSC expressing the SC marker CD105 release TEX that trigger angiogenesis and greatly enhanced lung metastases. The CD105⁺ TEX are characterized by sets of mRNAs and microRNAs supporting angiogenesis and tumor progression^[95]. Also melanoma TEX home to sentinel lymph nodes imposing molecular signals that support melanoma cell recruitment, extracellular matrix deposition, and vascular proliferation, thereby facilitating lymphatic metastasis^[485]. A proteome analysis of CoCa TEX uncovered enrichment particularly of metastasis-promoting factors (c-Met, S100A8, S100A9, tenascinC), of signal transduction molecules (ephrinB2, jagged1, src, TRAF2 and NCK interacting kinase <TNIK>), and lipid raft/lipid raft-associated components (caveolin, flotilin1 and 2, CD133) in TEX derived from a metastatic line. An additional key finding was the recovery of EpC-cld7 and TNIK-rap2A complexes in TEX^[132] (Figure 6C). The mode of TEX-induced premetastatic niche formation was also elaborated using TEX from a metastatic and a non-metastatic melanoma line. TEX from highly metastatic melanoma line increased the metastatic behavior of primary tumors by affecting bone marrow progenitors through c-Met. Melanoma-TEX reprogrammed bone marrow progenitors towards a provasculogenic phenotype defined by c-Kit, Tie2 and c-Met expression. Reduced c-Met expression in TEX diminished the pro-metastatic behavior of bone marrow cells. c-Met⁺/c-Kit⁺/Tie2⁺ bone marrow progenitors were also recovered in patients with metastatic melanoma. Premetastatic niche promoting TEX were high in $\alpha 4$, HSP and c-Met. The authors conclude that metastasizing melanoma TEX “home” to the bone marrow, where they reprogram bone marrow cells to support tumor growth and metastasis^[486]. The pathway of persisting reprogramming remains to be elaborated. However, it is conceivable that TEX contain transcription factors inducing a differentiation switch in this non-differentiated cells. In a mouse model of PaCa that metastasizes to the liver, TEX induce liver premetastatic niche formation and increase liver metastatic burden. TEX uptake by Kupffer cells promoted TGF β and FN secretion. The fibrotic microenvironment enhanced recruitment of bone marrow-derived macrophages. The authors report on high macrophage migration inhibitory factor (MIF) expression in PaCa TEX, where a MIF blockade prevented liver pre-metastatic niche formation and metastasis. High MIF expression in TEX was also seen at an early stage of PaCa growth in patients that developed liver metastasis^[101]. Evidence for the transfer of c-Met and for TEX stimulated c-Met-signaling in target cells fits to the CD44v6-c-Met complex recovery in Pa-CSC TEX^[202,433]. Activation of src also may well proceed *via* CD44v6-c-Met as well as *via* integrin tetraspanin complexes^[130,207]. The high recovery of inflammatory HSP in TEX could be due to the association with Tspan8 and could strengthen the

efficacy of the frequently described upregulation of chemokines and mostly immunosuppressive cytokines as well as of inflammatory complement components and S100 in Pa-CSC TEX^[487-490]. However, for the latter set of molecules a link to Pa-CSC markers remains to be defined (Figure 6C and D).

The miRNA content of TEX from CD44v6^{kd}, Tspan8^{kd} and cld7^{kd} cells differ from that of wt cell-derived TEX. Exploring the impact of CD44v6-linked miRNA transferred into stroma cells revealed 18 mRNA downregulation. From the total TEX miRNA, 60% could potentially be engaged in targeting these 18 mRNA. We focused on abundant miR-494, potentially targeting MAL and cdh17, and miR-542-3p, targeting cdh17 and TNF receptor associated factor 4 (TRAF4). MAL can contribute to differentiation and apical sorting^[491] and cdh17 to tumor growth/Wnt signaling^[492]; TRAF4 exerts morphogenetic functions^[493]. Lymph node stroma transfection with these miRNAs was accompanied by down-regulation of the predicted target(s). Significant up-regulation of mRNA in exosome-treated LnStr pointed toward mRNA up-regulation through miRNA silencing regulatory mRNA. Cdh17 represses MMP2 and MMP9 expression^[494] and down-regulation of cdh17 in miR-494 and miR-542-3p transfected stroma cells was accompanied by MMP2, MMP3 and MMP14 up-regulation^[202]. In another study with PaCa TEX, the authors found down-regulation of exosomal miR-155 and miR-196a and upregulation of miR-17-5p, upregulation correlating with metastasis and advanced tumor stages^[33]. Further controlling for the impact of miR-155 in PaCa TEX revealed normal fibroblasts to become converted into CAF after uptake of miR-155 containing TEX. TP53INP1 is a target of miR-155 in fibroblasts and TP53INP1 protein downregulation can contribute to fibroblasts activation^[495] (Figure 6E).

Without question TEX account for preparing a niche for migrating tumor cells. In PaCa TEX, there is strong evidence for a direct engagement of CD44v6, c-Met, integrins and Tspan8-associated integrins. An active contribution of cld7, the EpCAM-cld7 complex, CD133 and CXCR4 remains to be explored. According to the current state of knowledge, binding-induced as well as uptake-initiated signal transduction and the transfer of miRNA cooperate in target cell modulation.

Exosomal pancreatic cancer stem cell markers and the crosstalk with non-cancer stem cells

CSC TEX also modulate other tumor cells *via* protein, mRNA and miRNA transfer^[496,497].

One of the first and most impressive reports on TEX-uptake being critical in tumor growth stimulation describes the intercellular transfer of the oncogenic receptor EGFRvIII *via* TEX to glioma cells, lacking this receptor, which causes transformation of indolent glioma cells^[133]. The oncogenes Ras, Myc, SV40T also induce signaling and gene expression^[136,140,498]. Amphiregulin is an EGFR ligand. Compared to recombinant

protein, TEX-associated amphiregulin increases tumor invasiveness 5-fold. The finding strongly suggests the transfer of additional messages *via* TEX^[499]. In lung cancer TEX miR-21 and miR-29a act a TLR ligand and function as agonist. This leads to NFκB activation and IL6 and TNFα secretion promoting metastasis^[500]. A set of miRNA, including miR-584, miR-517c, are not detected in the donor cell, but are highly enriched in hepatocellular carcinoma TEX. A potential target of these miRNA is TGFβ activated kinase 1, which activates JNK and MAPK pathways and NFκB. In cocultures, these TEX miRNA promote anchorage-independent growth and apoptosis resistance^[501]. Apoptosis resistance can also rely on the transfer of multidrug resistance (MDR)1^[502], which is enriched in TEX^[503].

Furthermore, after oncogenic H-Ras-induced EMT, the TEX profile significantly changes, including TGFβ, TNFα, IL6, TSG101, Akt, ILK1, β-catenin, hepatoma-derived growth factor, casein kinase II, annexinA2, α3 integrin, caveolin and MMPs, the authors pointing out that the protein content of EMT TEX likely can induce EMT in recipient cells^[473]. TEX also contain EBV-derived LMP1, which modulates together with HIF1α EMT marker expression in recipient cells^[504]. TEX from human CSC-enriched CoCa lines can induce EMT in the CSC-depleted population. There is a strong induction of Notch, N-cadherin becomes upregulated and E-cadherin downregulated^[273]. In a rat PaCa, CSC TEX promote Notch and Snail transcription depending on the presence of Tspan8^[130] (Figure 6F). Still being at the descriptive level, it is obvious that CSC TEX can confer CSC features towards non-CSC including EMT, where TEX components work in concert.

Thus, Pa-CSC TEX markers are essential components for TEX targeting and account for selective responses, *e.g.*, *via* src activation. For other responses, including the impact of miRNA, a contribution of Pa-CSC markers to the recruitment into TEX was repeatedly demonstrated. Though information on the contribution of exosomal Pa-CSC markers on target cell activation/reprogramming is still limited, available data convincingly demonstrate the engagement of Pa-CSC markers in TEX assembly, binding and message transfer.

CONCLUSION

CSC markers have long been considered as a tool for CSC enrichment and diagnosis. We here demonstrate for the Pa-CSC markers CD44v6, c-Met, Tspan8, α6β4, EpCAM, clid7, CXCR4 and CD133 that these markers contribute in maintaining CSC features essential for tumor persistence and progression. These Pa-CSC markers are required to (1) maintain the CSC status by their engagement in signal transduction, transcription including miRNA and repression of tumor suppressor genes; (2) establish a stem cell niche including a premetastatic niche by affecting

via ligand binding target cell activation and message transfer; (3) support EMT by gene transcription and/or silencing; and (4) tumor cell migration and invasion *via* associated integrins and proteases.

The power of these markers relies on their residence in GEM, which allows for concerted activity and the engagement in TEX biogenesis and delivery. The CSC marker panel being maintained in TEX guarantees CSC to prepare the host for their maintenance at distant sites.

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Pulmonary complications of hepatic diseases

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Abstract

Severe chronic liver disease (CLD) may result from portal hypertension, hepatocellular failure or the combination of both. Some of these patients may develop pulmonary complications independent from any pulmonary pathology that they may have. Among them the hepatopulmonary syndrome (HPS), portopulmonary hypertension (PPH) and hepatic hydrothorax (HH) are described in detail in this literature review. HPS is encountered in approximately 15% to 30% of the patients and its presence is associated with increase in mortality and also requires liver transplantation in many cases. PPH has been reported among 4%-8% of the patient with CLD who have undergone liver transplantation. The HH is another entity, which has the prevalence rate of 5% to 6% and is associated in the absence of cardiopulmonary disease. These clinical syndromes occur in similar pathophysiologic environments. Most treatment modalities work as temporizing measures. The ultimate treatment of choice is liver transplant. This clinical review provides basic concepts; pathophysiology and clinical presentation that will allow the clinician to better understand these potentially life-threatening complications. This article will review up-to-date information on the pathophysiology, clinical features and the treatment of the pulmonary complications among liver disease patients.

Key words: Portopulmonary hypertension; Hepatopulmonary syndrome; Cirrhosis; Hepatocellular failure; Hepatic hydrothorax; Intrapulmonary shunting

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Core tip: Pulmonary complications are found in some patients with liver disease. The hepatopulmonary syndrome is found in 15% to 30% and, its presence, increases mortality and risk of requiring liver transplan-

tation. Portopulmonary hypertension has been reported to be present in 4% to 8% in patients who have undergone liver transplant evaluation. Hepatic hydrothorax, with a prevalence of 5%-6% in these patients, is suspected when a patient develops pleural effusions without presence of cardiopulmonary disease. All of these entities can only be solved with successful liver transplant.

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INTRODUCTION

Patients with chronic liver disease (CLD) can develop extra-hepatic pulmonary complications^[1]. Among them, three life-threatening conditions are of concern. The hepatopulmonary syndrome (HPS), in which there is vasodilatation of the microvascular vessels of the lungs, with or without the presence of hypoxemia in patients where other cardiopulmonary conditions has been excluded^[2]. Portopulmonary hypertension (PPH) results from arterial vasoconstriction linked to remodeling of the vascularity of the lung due to prolonged portal hypertension, which causes pulmonary arterial hypertension^[3]. This entity is rare, but when present is seen in females and patients with autoimmune hepatitis. Lastly, hepatic hydrothorax (HH) is a more common clinical entity that is suspected when a pleural effusion is present in patients with liver disease in the absence of cardiopulmonary conditions^[4].

This article reviews existing and up-to-date information on the epidemiology, pathophysiology, clinical manifestations, diagnosis and treatment options for these patients.

EPIDEMIOLOGY

Cirrhosis is the final pathway of CLD. In the United States, this condition has a prevalence of approximately of 0.27%^[5]. The prevalence is higher among non-Hispanic blacks, and Mexican Americans^[5]. Despite advances in its management, it has a reported mortality of 26.4% per-2-year interval compared with 8.4% in propensity matched-controls and the highest mortality in Latin America is found in Mexico^[5,6]. The HPS is found in approximately 15% to 30% of patients who has cirrhosis. HPS is infrequent among smokers^[7,8]. On the other hand, PPH has an estimated prevalence of 2% to 5% of patients with portal hypertension, and 4% to 6% in patients that are evaluated for liver transplant^[9]. The prevalence of HH, in patients with cirrhosis is approximately 4% to 6%^[10].

PATHOPHYSIOLOGY

In the HPS, there is excessive vasodilatation of pre-capillary and post-capillary vasculature, resulting in impaired oxygenation of venous blood as it passes through the lung, is the primary pathological insult^[11]. Several human studies have demonstrated the increase in nitric oxide production among these patients^[12,13]. This is thought to be related to shear stress and the production of endothelin-1 and tumor necrosis alpha (TNF α) in the liver, which in turn, activates the endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) in the lungs^[14]. Both, eNOS and iNOS, contribute to the monocyte accumulation of the Beta-Endothelin (ET β) receptor overexpression, in the pulmonary vascular endothelium^[15-17]. Other factors that can contribute to this monocyte accumulation include bacterial translocation and endotoxemia^[18]. The endothelial activation of the fractalkine chemokine (CX3CL1) in the lungs is a common pathway as it pertains to monocyte adherence in the pulmonary microcirculation^[17,19]. It has been postulated that this activation is part of the pathway of angiogenesis^[20]. In addition, another factor in activating eNOS and iNOS, is the increased carbon monoxide (CO) production in monocytes^[17,21]. Once these processes occur, the monocytes start to bind growing factors, such as the vasculo-endothelial growth factor-A (VEGF-A), causing angiogenesis and activating angiogenic signaling pathways^[17,20,22]. The more angiogenesis, the more intravascular monocytes, and finally an excessive vasodilatation (Figure 1).

Definition

PPH is defined in patients with CLD as pulmonary arterial hypertension with mean pulmonary artery pressure (MPAP) > 25 mmHg at rest or MPAP > 30 mmHg with exercise and pulmonary capillary wedge pressure (PAOP) of < 15 mmHg and pulmonary vascular resistance > 240 dynes·s·cm⁻⁵^[9,17,23]. The pathogenesis is not fully understood, as this entity has a low prevalence^[24,25]. Some authors describe the histopathology of PPH identical to that of idiopathic pulmonary arterial hypertension^[3,26]. In both instances, there is vascular injury caused by shear stress and vasoactive mediators (Endothelin-1, prostacyclin and thromboxane)^[9,17,27]. Moreover, these promote inflammatory processes with establishment of plexogenic arteriopathy, which advances to concentric intimal fibrosis, and smooth muscle hyperplasia and hypertrophy^[28]. Patients with autoimmune liver disease can develop PPH, but its relationship is not clear, and it is possible that these diseases should be regarded as systemic multi-organ manifestations^[29-31].

Pathogenesis

The most accepted pathogenesis for HH is the direct passage of ascitic fluid from the peritoneal cavity to

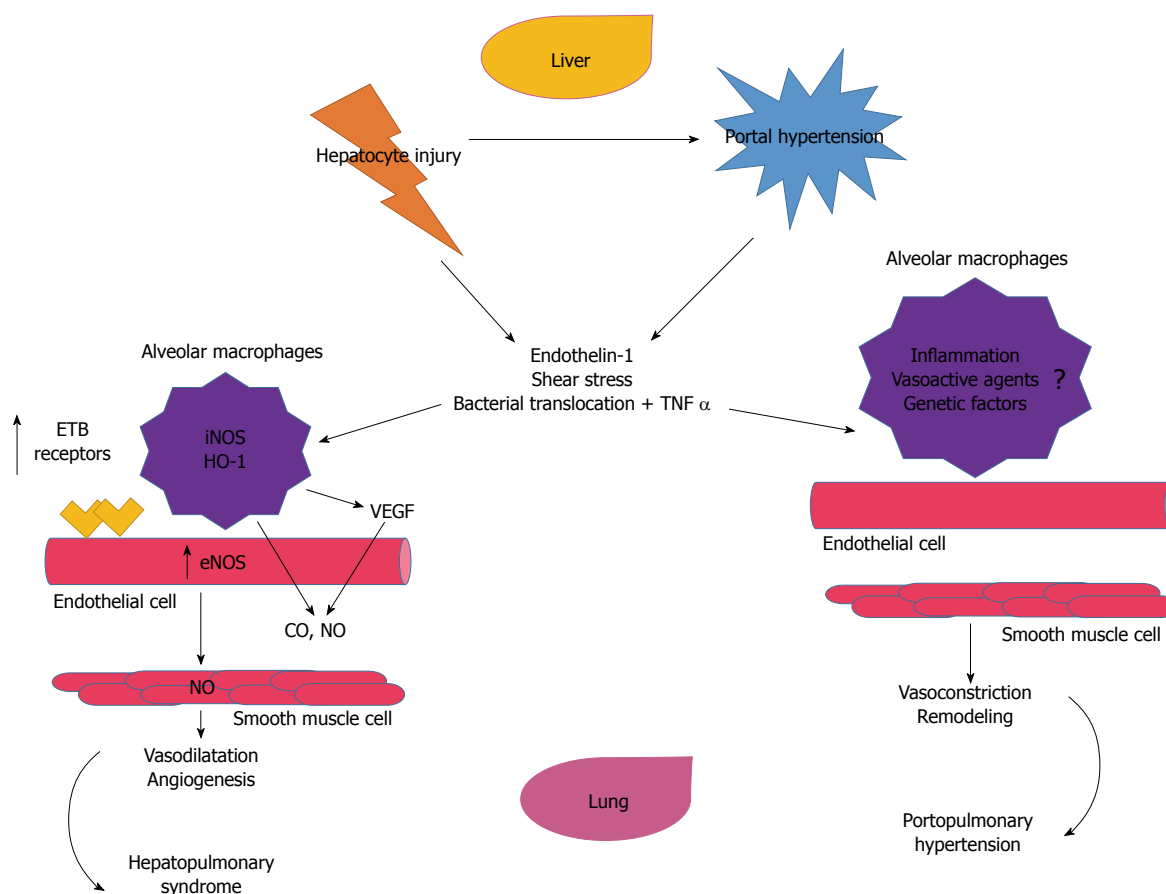


Figure 1 Pathophysiology of the hepatopulmonary syndrome and portopulmonary hypertension. TNF α : Tumoral necrosis factor alpha; ET_B: Endothelin type B; iNOS: Inducible nitric oxide synthase; HO-1: Heme oxygenase 1; VEGF: Vascular endothelial growth factor; eNOS: Endothelial nitric oxide synthase; CO: Carbon monoxide; NO: Nitric oxide.

the pleural space due to defects in the diaphragm^[4]. This is possible with a “valvular” mechanism, when the negative intra thoracic pressure favors the transfer of fluid across the defects^[30]. Some risk factors for HH include anatomic thinning and separation of tightly drawn collagenous fibers in the tendinous portion of the diaphragm (includes congenital factors), cirrhotic cachexia secondary to protein malnutrition, an increased in the intra abdominal pressure due to ascites that causes the peritoneum lining to evaginate, which results in formation of pleuroperitoneal blebs that are likely to rupture with unidirectional migration of ascitic fluid into the pleural cavity^[10,30,32]. Most of these defects measure less than 1 centimeter in diameter^[30]. Most of the effusions are right sided, in close to 85% of patients with HH, due to the fact that the tendinous portion of the diaphragm predominates^[32,33]. Bilateral effusions occur in only 2% of these patients^[34]. Other theories involve: the azygous vein, which increases its pressure and flow, leading to subsequent leakage of plasma; the movement of peritoneal fluid to the pleural space via transdiaphragmatic lymphatics; the leakage of the thoracic duct; and the decrease of the colloid-osmotic pressure due to hypoalbuminemia^[35-37]. Occasionally, some patients with CLD without ascites, may develop HH, as a result of the one way or unidirectional flow of

the ascitic fluid into the pleural space, exceeding the capacity of the pleura to resorb ascites^[38].

CLINICAL MANIFESTATIONS

In patients with CLD, the pulmonary complications may be subtle or life-threatening^[1,39]. These patients may have concurrent pulmonary conditions, such as chronic pulmonary obstructive disease.

HPS

Most patients are asymptomatic. However, 18% of HHS patients may have a clinical presentation that starts with an insidious onset of dyspnea during the early stages of the syndrome^[2,40]. Platypnea, which is worsening dyspnea during standing, or orthodeoxia (defined as hypoxemia that is exacerbated in the upright position), are seen in symptomatic patients with HPS^[2,11,12]. The cutoff value for orthodeoxia is defined as decrease in PaO₂ of 5% or 4 mmHg from the supine position^[17,41]. This hypoxemia is caused due to vasodilatation at the lower lobes of the lungs and an increased shunting through these regions when the patient is in the upright position^[42]. Significant desaturation during sleep may occur in these patients, even if the daytime hypoxemia is moderate^[11,43].

Patients with severe HHS, may display digital clubbing as well as cyanosis^[11,44].

PPH

Most patients complain of is dyspnea, that may be accompanied with orthopnea, fatigue, syncope, chest pain (that patients refer as oppressive), and lightheadedness^[9,45,46]. On physical examination, a tricuspid regurgitation murmur, with a pronounced P₂ sound can be heard. In addition, increased jugular venous pressure, peripheral edema and ascites^[9,47,48]. PPH is classified based on the MPAP values into mild (25-35 mmHg), moderate (35-50 mmHg), and severe (> 50 mmHg)^[46,49].

HH

When a patient with CLD develops a pleural effusion, we must suspect HH, even if ascites is absent^[38,50,51]. As noted, these patients could be totally asymptomatic or can exhibit symptoms of dyspnea on exertion, dry and non-productive cough, chest discomfort, hypoxemia and even respiratory failure^[37,52,53]. Relatively small amounts of fluid in the thoracic cavity (e.g., 1-2 L) can cause symptoms. Occasionally, patients with HH may have life-threatening complications such as acute tension hydrothorax (TH). In patients with TH, severe dyspnea and hypotension are seen^[52,53]. This process occurs rapidly, usually over the course of an hour, and be secondary to sudden pleuroperitoneal bleb^[50,54,55]. Spontaneous bacterial empyema (SBE), which is under-recognized because it is rarely described, may occur in 2% of cirrhotic patients, and 13%-16% among cirrhotic patients with hepatic hydrothorax^[10,34,56]. SBE may be confused with pleural empyema, as there is no evidence of abscess formation in the chest cavity and is commonly related to pneumonia^[10,56,57]. This clinical entity has a mortality rate of 20% to 38% and rapid diagnosis is essential for appropriate and timely initiation of treatment^[56,58].

DIAGNOSIS

HPS

The criterion for the diagnosis of HPS requires the presence of gas exchange abnormalities (due to intrapulmonary vasodilatation), and this can be measured with arterial blood gases, calculation of Alveolar-arterial oxygen gradient (abnormal if > 15-20 mmHg, on room air; age corrected) with or without hypoxemia (PaO₂). All of this is measured in the sitting position^[11,26]. In addition, contrast-enhanced echocardiography can be used to screen for intrapulmonary vasodilatation^[59]. When intrapulmonary shunting is present, the left ventricle gets opacified with the contrast at least 3 heartbeats after the right ventricle (delayed shunting)^[60].

Once the diagnosis of HHS is made it is important to classify its severity depending on the changes in

partial pressure of oxygen, into: mild with PaO₂ > 80 mmHg, moderate > 60 mmHg to < 80 mmHg, severe > 50 mmHg to < 60 mmHg or very severe < 50 mmHg^[12]. Pulmonary angiography may assist in the diagnosis and two patterns are commonly seen: First pattern seen has a finely diffuse, spidery arterial vascular abnormalities due to diffuse spongy appearance, and the second one has discrete, localized arteriovenous communications^[61]. As this is an invasive procedure, this is not commonly performed. A computed tomography (CT) of the chest can be useful, but it is less sensitive^[1,62].

PPH

Transthoracic echocardiography (TTE) has been deemed as one of the most practical method to detect PPH^[45]. This diagnostic technique attempts to identify the tricuspid regurgitant peak velocity, which helps in estimating the right atrial pressure by inferior vena cava changes on inspiration, and by using the modified Bernoulli equation, estimates the right ventricular systolic pressure in over 80% of patients with portal hypertension^[3]. It has a high sensitivity and specificity if the pressure of the right ventricle is > 40 mmHg. In that case the sensitivity in the diagnosis of PPH is 100% and the specificity is 82%^[63]. The American Association for the Study of Liver Disease recommends TTE to detect pulmonary hypertension in all the patients that are considered for liver transplant in the United States^[45]. These patients may present right-sided systolic dysfunction and finally cor pulmonale.

HH

A simple chest X-ray can be used to confirm pleural effusions, and a thoracentesis can then be performed to confirm the presence of peritoneal fluid^[10]. In the later test cell count, gram stain, culture, protein, albumin, lactate dehydrogenase and bilirubin are commonly analyzed. The pleural fluid composition in hepatic hydrothorax is usually transudative, however, due to a difference in the water reabsorptive capacity between the pleural space and peritoneal cavity, it has a higher protein concentration than ascitic fluid. If excessive diuresis is used, this fluid can be exudative^[38]. In these patients, a CT of the chest can be helpful in differentiating and eliminating the pulmonary or pleural pathologies of left-sided pleural effusions^[55]. Ultrasonography and magnetic resonance can be utilized to visualize diaphragmatic defects^[32,50].

TREATMENT

HPS

To date, there is no effective medical therapy for this clinical condition. Spontaneous resolution is quite rare. In uncontrolled clinical trials, the use of beta-blockers, steroids, cyclophosphamide and/or nitric oxide have shown no mortality benefit^[2,12,23,26]. As the mortality

Table 1 Pulmonary hypertension; drug classification

| Classification | Name | Mechanism of action |
|--------------------------------|--------------|--|
| Endothelin receptor antagonist | Bosentan | Dual ETA and ETB receptor subtypes antagonist. Specifically, inhibition of ET-1 receptors |
| | Ambrisentan | Highly selective ETA receptor inhibition |
| | Macitentan | High affinity ETA than ETB antagonist. |
| Phosphodiesterase 5 inhibitors | Sildenafil | High selectivity for PD5 <i>vs</i> PD2, 3 and 4. |
| | Tadalafil | High selectivity for PD5 compared with PD1, 4, 7 and 10. |
| Prostanoids | Epoprostenol | Synthetic prostacyclin with potent effects of vasodilatation and platelet aggregator inhibitor. |
| | Treprostinil | Long acting tricyclic benzindene synthetic analogue of prostacyclin. Vasodilator and inhibits platelet inhibition. |

Table 2 Differences between the hepatopulmonary syndrome and portopulmonary hypertension

| | Hepatopulmonary syndrome | Portopulmonary hypertension |
|-------------------|---|--|
| Pathophysiology | Severe vasodilatation Production of endothelin-1 and tumor necrosis alpha and eNOS Increase of CO Vasculoendothelial growth factor-A Angiogenesis | Severe vasoconstriction Concentric intimal fibrosis, and smooth muscle hyperplasia and hypertrophy Endothelin-1, prostacyclin and thromboxane |
| Clinical features | Most patients are asymptomatic Dyspnea Platypnea Orthodeoxia Significant sleep-time oxygen desaturation | Dyspnea Orthopnea Fatigue Syncope Chest pain Lightheadedness Tricuspid regurgitation murmur, with a pronounced P2 sound Increased jugular venous pressure Peripheral edema Ascites |
| Diagnosis | Corrected alveolar-arterial oxygen gradient (Abnormal if > 15-20 mmHg) with or without hypoxemia (PaO ₂), all in sitting position Contrast-enhanced echocardiography Degree of severity: Alveolar-Arterial oxygen gradient > 15 mmHg, mild with PaO ₂ > 80 mmHg, moderate > 60 mmHg to < 80 mmHg, severe > 50 mmHg to < 60 mmHg or very severe < 50 mmHg | Transthoracic echocardiography. |
| Treatment | Pulmonary angiography Liver transplant | Endothelin receptor antagonist, phosphodiesterase type-5 inhibitors, prostanoids, and combination therapy Sildenafil alone or combined with prostacyclins Transjugular intrahepatic portosystemic shunting Liver transplant |

eNOS: Endothelial nitric oxide synthase; CO: Carbon monoxide; NO: Nitric oxide.

rate is quite high in these patients, the only chance for clinical improvement is undergoing liver transplant. This therapeutic intervention is successful in up to 85% of the patients with HHS that undergo this life-saving technique^[11]. Priority of transplant is given to patients with HHS that also present hypoxemia (PaO₂ < 60 mmHg)^[64].

PPH

The primary goal in these patients is to reduce the obstruction of pulmonary artery blood flow, in an attempt to improve hemodynamics^[49-51]. This is accomplished by reducing the MPAP, and the pulmonary vascular resistance^[45]. A secondary goal is to normalize the right ventricular pressure^[3,25,29]. A variety of medications have been tried in these patients targeting

pulmonary arterial hypertension. Among them, endothelin receptor antagonist, phosphodiesterase type-5 inhibitors, prostanoids, and combination therapy have been utilized^[65,66]. Sildenafil as a single agent or in combination with prostacyclins is commonly used (Table 1)^[49,67]. These agents are usually used to improve symptoms, prior to liver transplantation^[63]. Failure to reduce the MPAP to < 50 mmHg, is considered a contraindication for transplant^[68].

Over the past two decades, transjugular intrahepatic portosystemic shunting (TIPS), has been used as a treatment for uncontrolled gastrointestinal bleeding, refractory ascites or hydrothorax in patients with CLD. It can temporarily increase the MPAP, carbon monoxide and PVR and clinicians must be vigilant of these changes^[69]. A contraindication to this procedure

is a right ventricular systolic pressure > 50 mmHg, as well as an abnormal right ventricular size and function^[25,49,68]. The pathophysiology, comparing the HPS and PPH is shown in Figure 1. Table 2 illustrates the pathophysiology, clinical features, diagnoses and treatment between these two diseases entity.

HH

Once the HH is managed emergently, these patients must be evaluated for liver transplant^[51]. The primary treatment objective is to reduce the ascitic fluid accumulation and to relieve symptoms. In addition, preventing complications is paramount in these patients^[4,30,37]. Sodium restriction is the first-line treatment, as well as gentle diuresis (e.g., spironolactone at a dose of 50-100 up to 400 mg per day)^[34,37,70]. Before using a second diuretic, the dose of spironolactone must be increased gradually. A low-sodium diet, with 70-90 mmol per day, and weight loss of 0.5 kg per day in patients without edema, and 1.0 kg per day in those with edema is an initial goal of therapy. Thoracentesis is helpful for immediate symptomatic relief, and it is indicated for large pleural effusions (1.5-2.0 L) and for those with refractory hydrothorax and patients who are not candidates for TIPS^[70,71]. As noted above, TIPS could be used to decompress the portal tract and decrease the portal venous pressure and can help in decreasing the rate of ascites formation. This intervention partially resolves the pathogenesis of ascites formation and it is a better alternative than that repeated thoracentesis^[72]. In these individuals, the indications for TIPS are the patients with refractory ascites, failure to respond to adequate diuretic therapy, and frequent thoracentesis (> 1 in a period of 2-3 wk)^[73]. TIPS can be done safely in patients < 65 years of age with a serum bilirubin < 3 mg/dL, ALT < 100 IU/L and a Child-Pugh score < 10^[31]. Other contraindications in the context of HH include hepatic encephalopathy, pulmonary hypertension and an ejection fraction < 60%^[74].

CONCLUSION

The pulmonary complications seen in patients with CLD frequently require liver transplant evaluation. The HPS is more frequent than PPH hypertension and HH, and the best chance for improved life-quality in these patients is liver transplant. PPH can be confused with idiopathic pulmonary hypertension. TIPS can be used in some of these patients. Of all the complications reviewed in this manuscript, HH has the best outcomes.

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Basic Study

Silybin counteracts lipid excess and oxidative stress in cultured steatotic hepatic cells

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Abstract

AIM: To investigate *in vitro* the therapeutic effect and mechanisms of silybin in a cellular model of hepatic steatosis.

METHODS: Rat hepatoma FaO cells were loaded with lipids by exposure to 0.75 mmol/L oleate/palmitate for 3 h to mimic liver steatosis. Then, the steatotic cells were incubated for 24 h with different concentrations (25 to 100 μ mol/L) of silybin as phytosome complex with vitamin E. The effects of silybin on lipid accumulation and metabolism, and on indices of oxidative stress were evaluated by absorption and fluorescence microscopy, quantitative real-time PCR, Western blot, spectrophotometric and fluorimetric assays.

RESULTS: Lipid-loading resulted in intracellular triglyceride (TG) accumulation inside lipid droplets, whose number and size increased. TG accumulation was mediated by increased levels of peroxisome proliferator-activated receptors (PPARs) and sterol regulatory element-binding protein-1c (SREBP-1c). The lipid imbalance was associated with higher production of reactive oxygen species (ROS) resulting

in increased lipid peroxidation, stimulation of catalase activity and activation of nuclear factor kappa-B (NF- κ B). Incubation of steatotic cells with silybin 50 μ mol/L significantly reduced TG accumulation likely by promoting lipid catabolism and by inhibiting lipogenic pathways, as suggested by the changes in carnitine palmitoyltransferase 1 (CPT-1), PPAR and SREBP-1c levels. The reduction in fat accumulation exerted by silybin in the steatotic cells was associated with the improvement of the oxidative imbalance caused by lipid excess as demonstrated by the reduction in ROS content, lipid peroxidation, catalase activity and NF- κ B activation.

CONCLUSION: We demonstrated the direct anti-steatotic and anti-oxidant effects of silybin in steatotic cells, thus elucidating at a cellular level the encouraging results demonstrated in clinical and animal studies.

Key words: Non-alcoholic fatty liver disease; Steatotic hepatocytes; Silybin; Lipid metabolism; Oxidative stress; Lipid droplets; Mitochondrial β -oxidation

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Core tip: FaO hepatic cells loaded with lipids by exposure to oleate/palmitate mixture represent a widely accepted cellular model of hepatic steatosis. FaO steatotic cells were used to investigate *in vitro* the possible direct effects of silybin as phytosome complex with vitamin E on lipid accumulation and metabolism and on oxidative stress. We demonstrated the ability of silybin in reducing fat accumulation and improving the oxidative imbalance caused by lipid excess. The results may elucidate at a cellular level the encouraging results demonstrated for silybin in previous clinical and animal studies.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is characterized by excess fat accumulation, mainly in the form of triglycerides (TGs), in hepatocytes of subjects who do not consume excess alcohol. NAFLD definition encompasses a large spectrum of liver abnormalities which range from the simple steatosis, to non-alcoholic steatohepatitis (NASH), till to cirrhosis and hepatocellular carcinoma^[1,2]. NAFLD is the most common cause of abnormal liver function tests in Western countries, and evidences substantiate a

strong association between NAFLD and metabolic abnormalities such as obesity, insulin resistance, and metabolic syndrome^[3].

In the hepatocyte, TGs^[4] are synthesized from fatty acids (FAs) deriving from three major sources: plasmatic non-esterified fatty acids (NEFAs) from adipose tissue, *de novo* lipogenesis, and dietary FAs^[5]. Uptake of FAs by hepatocytes is related to their serum concentrations and is mediated by different classes of fatty acid transport proteins^[6]. In the liver, FAs follow three different destinations: (1) oxidation, mainly in mitochondria, but also in extra-mitochondrial organelles such as peroxisomes; (2) assembly and export as very low-density lipoproteins (VLDL); and (3) storing as TGs within lipid droplets (LDs).

Storing of excess TGs in LDs is a protective mechanism against FA-induced toxicity that is mainly related to FA oxidation. Most FAs are metabolized through β -oxidation in mitochondria which is primarily regulated by carnitine palmitoyltransferase 1 (CPT-1) required for transport of long-chain fatty acids into mitochondria^[7]. Over-active FA oxidation leads to over-production of reactive oxygen species (ROS) with consequent oxidative stress^[8,9]. Excess ROS as well as pro-inflammatory cytokines can activate inflammatory signaling such as that sustained by the transcription factor nuclear factor kappa-B (NF- κ B) which is implicated in the response to oxidative stress^[10].

In light of these considerations, a reduction in liver steatosis through a stimulation of lipolytic pathways may potentially expose hepatocytes to the damaging effect of excess free FAs^[11] and this point has to be considered when anti-steatotic molecules are tested for therapeutic applications.

Medicinal plants have become popular as the source of dietary supplements^[12]. Flavonoids, a large class of polyphenolic products, are well-known antioxidants and free radical scavengers^[13]. Silymarin, the standardized extract from milk thistle (*Silybum marianum*), and its major active compound silybin, have been used for a long time as a hepatoprotective agents for the treatment of acute and chronic liver diseases^[14]. Previous clinical findings evidenced the efficacy of silybin on insulin resistance and liver injury in patients with NAFLD^[15]. Moreover, in a recent study on sixty four patients with NASH, silymarin helped to lower the hepatic enzymes, particularly ALT^[16]. The improvement of liver histology after silybin treatment was recently reported in a multicenter randomized controlled trial^[17]. However, the molecular mechanisms associated with the hepatoprotective activity of silybin remain to be elucidated.

The hepatic lipid metabolism is governed by two main families of transcription factors, the peroxisome proliferator-activated receptors (PPARs) that regulate both lipogenic and lipolytic pathways^[18], and the sterol regulatory element-binding proteins (SREBPs) that stimulate sterol and fatty acid biosynthesis^[19,20]. FAs are endogenous ligands of all PPAR isoforms^[6];

uptake of FAs into hepatocytes and their oxidation is regulated mainly by PPAR α , while their esterification and conversion to TGs by PPAR γ and SREBP-1, whose expression typically increases in NAFLD^[21]. Moreover, macroautophagy, a process that leads to the degradation of cellular constituents through lysosomes^[22], participates in lipid metabolism through the breakdown of LDs (lipophagy). A decreased autophagic function may promote the development of hepatic steatosis and the progression of steatosis to NASH^[23]. The autophagy-related protein 7 (Atg7) is considered essential for this process in mammalian cells^[23].

This study aimed to clarify whether silybin as phytosome complex with vitamin E may favorably affect lipid and radical homeostasis using an *in vitro* model of NAFLD induced by the exposure of hepatoma FaO cells to exogenous FAs. Changes in expression of PPARs and SREBP-1c, the main regulators of lipid metabolism, as well as of CPT1, the master controller of mitochondrial FA oxidation, and *Atg7*, a key autophagy-promoting gene, have been assessed to investigate the pathways possibly activated by silybin. In parallel, the stimulation of defense systems against oxidative stress, such as catalase and NF- κ B, were evaluated to assess the potential protective effects of silybin.

MATERIALS AND METHODS

Chemicals

All chemicals, unless otherwise indicated, were of analytical grade and were supplied by Sigma-Aldrich Corp. (Milan, Italy).

Cell treatments

Rat hepatoma FaO cells (European Collection of Cell Cultures, Sigma-Aldrich Corp., Milan, Italy) are a liver cell line maintaining hepatocyte specific markers^[24]. Cells were grown in Coon's modified Ham's F12 with 10% foetal bovine serum (FBS). For treatments, cells were grown until 80% confluence, then incubated overnight in high-glucose medium with 0.25% bovine serum albumin (BSA). Steatosis was induced by exposing cells for 3 h to an oleate/palmitate mixture (2:1 molar ratio, final concentration 0.75 mmol/L). Thereafter, steatotic cells were incubated for 24 h with 0 to 100 μ mol/L (final concentration) of silybin (S) as phytosome complex with vitamin E (Realsil[®], Istituto Biochimico Italiano, Lorenzini S.p.a, Italy). Silybin stock (10 mmol/L) was prepared in dimethyl sulfoxide (DMSO) and then diluted with the culture medium to a final concentration 0.3 mmol/L.

The viability of FaO cells upon exposure to FAs or silybin, both as single agents or combined, was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and measured spectrophotometrically^[25].

Quantification of triglycerides

For determination of intracellular TG content, at the end of each treatment cells were scraped and centrifuged at 14000 $\times g$ for 3 min. After cell lysis, obtained by passing cell suspension through 25 gauge needle, lipids were extracted using the chloroform/methanol (2:1) method^[26] and TG content was measured by spectrophotometric analysis ("Triglycerides liquid" kit, Sentinel, Milan, Italy). Values were normalized to protein content as determined by the bicinchoninic acid (BCA) method using BSA as a standard^[27]. In parallel, TG content was determined in the culture medium. Data are expressed as percent TG content relative to controls.

Lipid droplet analysis

Cells were grown on collagen-coated glass slides (Falcon, BD, Milan, Italy); neutral lipids were visualized using the selective Oil-RedO (ORO) dye. Briefly, after fixing in 4% paraformaldehyde, cells were washed with PBS, stained with ORO 1% in triethyl phosphate 60% for 20 min and washed^[28]. Slides were examined by Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany). In parallel, the LD abundance inside the cells was assessed fluorimetrically using Nile Red (NR), a vital lipophilic dye. At the end of each treatment, cells were incubated with 0.3 μ mol/L NR solution (from a stock solution of 100 μ g/mL in PBS) for 30 min at 37 $^{\circ}$ C. After washing with PBS the NR fluorescence was measured by LS-50B spectrofluorimeter (Perkin Elmer, United States) at λ_{ex} = 580 nm and λ_{em} = 630 nm and slit width set to 5.0^[29]. All measurements were performed at 25 $^{\circ}$ C using a water-thermostated cuvette holder.

ROS production and lipid peroxidation measurement

The oxidation of the cell-permeant 2'-7' dichlorofluorescein diacetate (DCF-DA, Fluka, Germany) to 2'-7' dichlorofluorescein (DCF) is extensively used for quantifying *in situ* the production of H₂O₂ and other ROS^[30]. Stock solution of DCF-DA (10 mmol/L in DMSO) was prepared and stored at -20 $^{\circ}$ C in the dark. At the end of each treatment, cells were scraped and gently spun down (600 $\times g$ for 10 min at 4 $^{\circ}$ C). After washing, cells were loaded with 10 μ mol/L DCF-DA in PBS for 30 min at 37 $^{\circ}$ C in the dark, centrifuged and suspended in PBS. The DCF fluorescence was measured fluorometrically at λ_{ex} = 495 nm and λ_{em} = 525 nm using. All measurements were performed at 25 $^{\circ}$ C using a water-thermostated cuvette holder.

In parallel, H₂O₂ production was assessed directly on cells grown on collagen-coated glass slides. After treatment, cells were incubated with 100 μ mol/L DCF-DA for 1 h^[31]. Slides were examined by Leica DMRB light microscope equipped with a Leica CCD camera DFC420C. Lipid peroxidation was determined spectrophotometrically through the thiobarbituric

Table 1 Characteristics of the primer pairs used for reverse transcription-quantitative real-time PCR analysis

| Primer name | Primer sequence (5'→3') | Annealing temperature (°C) | Product lenght (bp) | Accession ID |
|------------------|-------------------------|----------------------------|---------------------|----------------|
| GAPDH-F | GACCCCTTCATTGACCTCAAC | 60 | 136 | DQ403053 |
| GAPDH- R | CGCTCCTGGAAGATGGTGATGGG | | | |
| PPAR α -F | CCCCACTTGAAGCAGATGACC | 60 | 139 | NM_013196 |
| PPAR α -R | CCCTAAGTACTGGTAGTCCGC | | | |
| PPAR δ -F | AATGCCTACCTGAAAACTTCAAC | 60 | 96 | AJ306400 .1 |
| PPAR δ -R | TGCCTGCCACAGCGTCTCAAT | | | |
| PPAR γ -F | CGGAGTCCTCCAGCTGTTCGCC | 60 | 116 | Y12882 |
| PPAR γ -R | GGCTCATATCTGTCTCCGTCTTC | | | |
| CPT1-F | CCGCTCATGGTCAACAGCA | 60 | 105 | NM_031559 |
| CPT1-R | CAGCAGTATGGCGTGGATGG | | | |
| Atg7-F | CCTCAGCGGATGTATGGACC | 60 | 160 | NM_001012097.1 |
| Atg7-R | AGCCACATTACACCCCAAGG | | | |

F: Forward sequence; R: Reverse sequence.

acid reactive substances (TBARS) assay which is based on the reaction of malondialdehyde (MDA; 1,1,3,3-tetramethoxypropane) with thiobarbituric acid (TBA)^[32]. Briefly, 1 vol. of cell suspension was incubated for 45 min at 95 °C with 2 vol. of TBA solution (0.375% TBA, 15% trichloroacetic acid, 0.25 mol/L HCl. Then, 1 vol. of N-butanol was added and the organic phase was read using a Varian Cary50 spectrophotometer at 532 nm. Results were expressed as pmol MDA/mL per milligram protein.

Determination of catalase activity

Catalase (CAT, EC 1.11.16) activity was evaluated in both 12000 × *g* supernatant and pellet of cell lysates following the consumption of H₂O₂ at 25 °C according to^[33]. Enzyme activity (as the sum of both pellet and supernatant) was expressed as μ moles of decomposed H₂O₂ per min/mg protein. Protein content was determined by BCA method. All measurements were performed at 25 °C using a water-thermostated cuvette holder.

RNA extraction and quantitative real-time PCR

RNA was isolated using the Trizol reagent, cDNA was synthesized and quantitative real-time PCR (qPCR) was performed in quadruplicate using 1 × IQTM SybrGreen SuperMix and Chromo4TM System apparatus (Biorad, Milan, Italy)^[34]. The relative quantity of target mRNA was calculated by the comparative C_q method using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene, and expressed as fold induction with respect to controls^[35]. Primer pairs were designed *ad hoc* starting from the coding sequences of *Rattus norvegicus* available on the GenBank database (<http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html>) and synthesized by TibMolBiol custom oligosynthesis service (Genova, Italy). Primers are listed in Table 1.

Western blotting

Both cellular and nuclear homogenates were processed by Western blot analysis to assess the protein levels

of SREBP1-c and NF- κ B/p65, respectively. In fact, activation of the NF- κ B transcription factor is associated with its phosphorylation and nuclear translocation of the p65 component of the complex. Cells were lysed on ice in lysis buffer (NaCl 150 mmol/L, Tris HCl pH 7.4, 50 mmol/L, SDS 0.33%) as described elsewhere^[34]. For nuclear extraction, the cellular pellet was suspended in 400 μ L ice-cold Buffer A (20 mmol/L Tris HCl pH 7.8, 50 mmol/L KCl, 10 μ g/mL Leupeptin, 0.1 mmol/L Dithiothreitol-DTT, 1 mmol/L phenylmethanesulfonyl fluoride-PMSF); then 400 μ L Buffer B (Buffer A plus 1.2% Nonidet P40) was added. The suspension was vortex-mixed for 10 sec; after centrifugation (14000 × *g* for 30 s, 4 °C) the supernatant was discarded and the nuclear pellet was washed with 400 μ L Buffer A and centrifuged. Next, the nuclear pellet was suspended in 100 μ L Buffer B, mixed thoroughly in ice for 15 min and finally centrifuged (14000 × *g* for 20 min, 4 °C). The supernatant containing the nuclear extracts was collected and the protein content was measured by BCA method. About 30-50 μ g proteins were electrophoresed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)^[36]. Membrane was blocked for 1 h in 5% fat-free milk/PBS (pH 7.4) and probed using mouse anti-human SREBP-1 (SC-13551) or rabbit anti-human NF- κ B p65 (SC-109) antibodies supplied by Santa Cruz Biotechnology (DBA, Milan, Italy). Membranes were incubated overnight at 4 °C with primary antibody in PBST buffer (PBS with 0.1% Tween 20)^[37], washed and incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Sigma-Aldrich), as a secondary antibody in PBST for 1 h at room temperature. Immune complexes were visualized using an enhanced chemiluminescence Western blotting analysis system (Bio-Rad ChemiDoc XRS System). Films were digitized and band optical densities were quantified against the actin band using a computerized imaging system and expressed as Relative Optical Density (ROD, arbitrary units). ROD of each band was expressed as percentage respect to control.

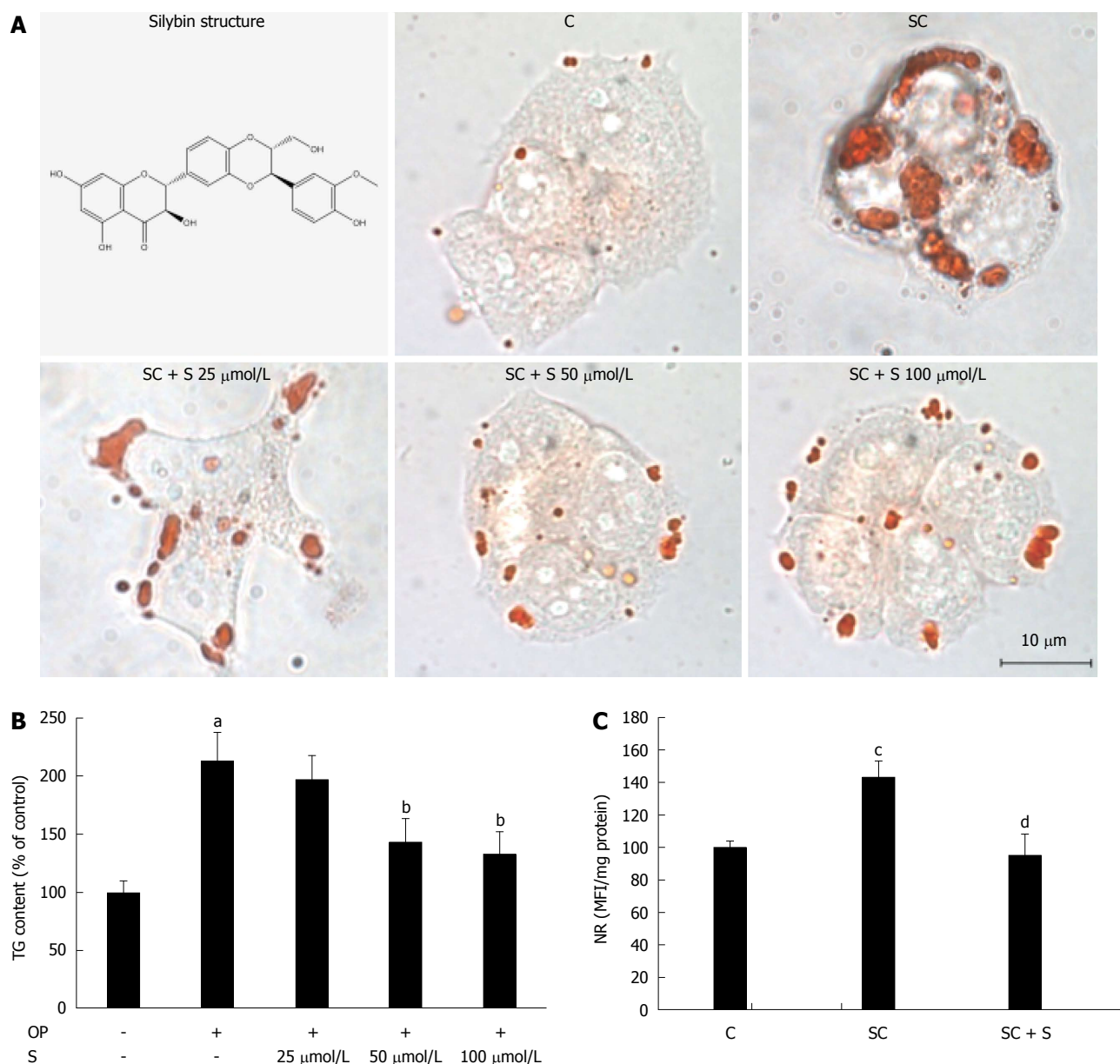


Figure 1 Lipid-lowering effects of silybin in steatotic FaO cells. A: Neutral lipids were visualized *in situ* by ORO-staining in control (C) and steatotic FaO cells incubated in the absence (SC) or in the presence (SC + S) of the silybin at different doses (silybin 25; 50; 100 μmol/L) (magnification × 40; Bar: 10 μm). Silybin structure is also shown; B: On the same samples TG content was quantified by spectrophotometric assay; data are expressed as percent TG content relative to control and normalized for total proteins; C: LD accumulation was evaluated by NR-staining in control and steatotic FaO cells incubated in the absence or in the presence of 50 μmol/L silybin; data are expressed as percent mean fluorescence intensity (MFI) relative to control and normalized for total proteins. Values are mean ± SD from a least three independent experiments. ANOVA followed by Tukey's test was used to assess the statistical significance between groups. Significant differences are denoted by symbols: ^a $P \leq 0.001$, ^c $P \leq 0.01$ C vs OP and ^b $P \leq 0.001$, ^d $P \leq 0.01$ OP vs silybin.

Statistical analysis

RNA and protein data are expressed as mean ± SD of at least four independent experiments in triplicate. Statistical analysis was performed using ANOVA with Tukey's post-test (GraphPad Software, Inc., San Diego, CA, United States).

RESULTS

Effects of silybin on lipid accumulation

Lipid accumulation was visualized *in situ* by optical microscopy of ORO-stained cells in control (C) and

steatotic hepatocytes incubated for 24 h in the absence (SC) or in the presence (SC + S) of increasing concentrations of silybin (25, 50, and 100 μmol/L). The number and size of LDs increased markedly in steatotic cells, and decreased dose-dependently with silybin incubation (Figure 1A). Also TG content was significantly higher in steatotic than in control cells (+113%; $P \leq 0.001$) (Figure 1B). Incubation with silybin 50 μmol/L and 100 μmol/L decreased significantly the TG content by -33% and by -38%, respectively, ($P \leq 0.001$ for both doses vs untreated steatotic cells). Silybin did not affect the lipid content

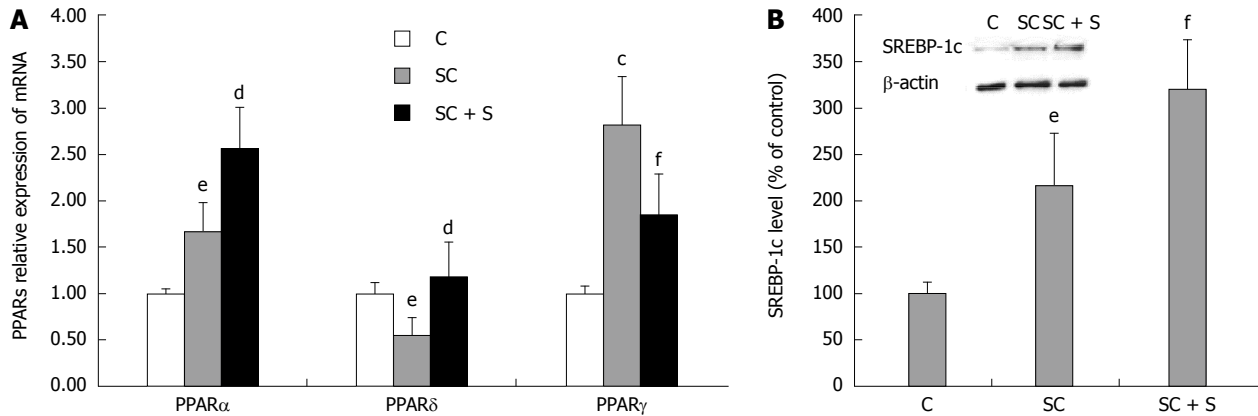


Figure 2 Effects of silybin on transcription factors regulating lipid metabolism. In control (C) and steatotic FaO cells incubated in the absence (SC) or in the presence (SC + S) of silybin 50 $\mu\text{mol/L}$ we assessed. A: mRNA expression of PPAR α , PPAR δ and PPAR γ by qPCR; GAPDH was used as the internal control for quantifying gene expression; data expressed as fold induction with respect to controls; B: Densitometric analysis of SREBP-1c by Western blotting; β -actin was the protein loading control used as housekeeping gene for normalization; data are expressed as percentage values with respect to controls. Values are mean \pm SD from at least three independent experiments. ANOVA followed by Tukey's test was used to assess the statistical significance between groups. Significant differences are denoted by symbols: $^aP \leq 0.01$, $^bP \leq 0.05$ C vs SC and $^cP \leq 0.01$, $^dP \leq 0.05$ SC vs Silybin.

of control cells (data not shown).

Following these pilot experiments, silybin at a concentration of 50 $\mu\text{mol/L}$ was used for all further experiments. Using NR fluorescence we measured LD accumulation as a function of the treatments. The average content of LDs increased significantly by +43% ($P \leq 0.01$) in steatotic cells compared to control, and decreased by -33% ($P \leq 0.01$) with silybin 50 $\mu\text{mol/L}$ compared to steatotic cells (Figure 1C). Cell viability was not significantly affected by FA and/or silybin treatments as single agents or combined (data not shown).

Effects of silybin on lipid metabolism pathways

Lipid metabolism pathways are controlled by PPARs, a family of FA-regulated transcription factors. In rat hepatocytes, we have previously showed the following abundance of the three PPAR transcripts: PPAR α > PPAR δ > PPAR γ ^[38]. The present results show that expression of PPAR α mRNA was 1.7 fold higher in steatotic cells compared to control ($P \leq 0.05$), and increased further upon incubation with silybin 50 $\mu\text{mol/L}$ (+54% with respect to steatotic cells; $P \leq 0.01$) (Figure 2A). A significant increase was also observed in PPAR γ mRNA expression upon lipid-loading (2.8 fold induction with respect to control; $P \leq 0.01$), but, in this case, silybin 50 $\mu\text{mol/L}$ reduced the up-regulation (-34% with respect to steatotic cells, $P \leq 0.05$) (Figure 2A). By contrast, mRNA expression of PPAR δ decreased upon lipid-loading (about 0.55 fold induction with respect to control; $P \leq 0.05$) and silybin 50 $\mu\text{mol/L}$ restored PPAR δ expression to values similar to control (+115% compared to steatotic cells, $P \leq 0.01$) (Figure 2A).

SREBP-1c is a PPAR α -target gene which regulates expression of lipogenic enzymes. Activation of SREBP-1c increased in steatotic hepatocytes (+116%; $P \leq 0.05$) with respect to control and further increased

upon exposure to silybin 50 $\mu\text{mol/L}$ (+48% compared to steatotic cells; $P \leq 0.05$) (Figure 2B).

A reduced TG accumulation in hepatocytes might be sustained by both stimulation of oxidative and/or secretory pathways. CPT-1, the main regulator of mitochondrial β -oxidation of FAs, is significantly up-regulated in steatotic cells upon incubation with silybin 50 $\mu\text{mol/L}$ (+217% with respect to steatotic cells; $P \leq 0.001$) (Figure 3A). At the same time, the mRNA expression of *Atg7*, an autophagy-promoting gene in hepatocytes, was significantly increased in steatotic cells (1.45 fold induction with respect to control; $P \leq 0.05$), but it did not change upon treatment with silybin 50 $\mu\text{mol/L}$ (Figure 3B). On the other hand, steatotic cells showed an increased TG secretion into the culture medium compared to control cells (+57%; $P \leq 0.001$), but incubation with silybin 50 $\mu\text{mol/L}$ did not further stimulate TG secretion as compared to steatotic cells (Figure 3C).

Effects of silybin on oxidative stress

The intracellular production of ROS, mainly hydrogen peroxide (H_2O_2), was visualized *in situ* by fluorescence microscopy of DCF-stained cells (Figure 4). Higher and diffuse DCF fluorescence was observed in steatotic cells (Figure 4C, D) and it was lower in cells treated with silybin 50 $\mu\text{mol/L}$ (Figure 4E, F). DCF fluorescence was almost null in control cells (Figure 4A). These changes were quantified by spectrofluorometer readings (Figure 4B) that showed a significant DCF decrease (-45%; $P \leq 0.001$) in silybin-treated cells with respect to steatotic cells used as control.

Also MDA level was greater in steatotic than in control cells (+100%; $P \leq 0.001$) and decreased with silybin 50 $\mu\text{mol/L}$ (-57% with respect to steatotic cells; $P \leq 0.001$) (Figure 5A). The analyses of antioxidant defence mechanisms showed increased catalase activity in steatotic cells with respect to control (+45%

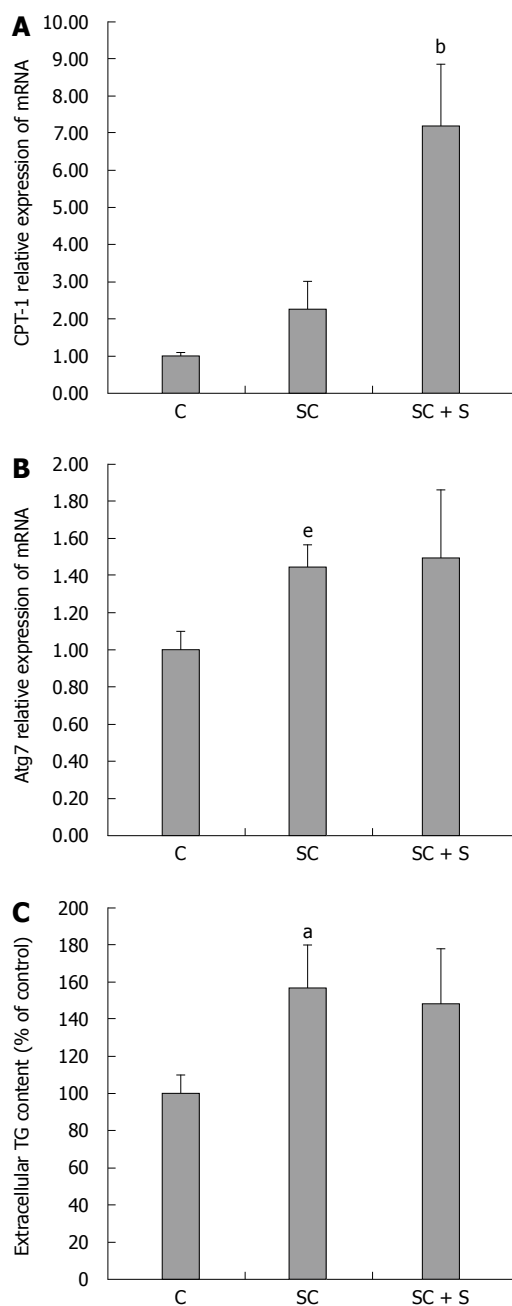


Figure 3 Effects of silybin on lipid catabolism pathways. In control (C) and steatotic FaO cells incubated in the absence (SC) or in the presence (SC + S) of silybin 50 $\mu\text{mol/L}$ we assessed: mRNA expression of CPT-1 (A) and of Atg7 (B) by qPCR; GAPDH was used as the internal control for quantifying gene expression and data expressed as fold induction with respect to controls; extracellular TG content quantified in the medium by spectrophotometric assay (C); data are expressed as percent TG content relative to control and normalized for total proteins; Values are mean \pm SD from at least three independent experiments. ANOVA followed by Tukey's test was used to assess the statistical significance between groups. Significant differences are denoted by symbols: $^aP \leq 0.001$, $^bP \leq 0.05$ C vs OP and $^cP \leq 0.001$, $^dP \leq 0.01$ OP vs Silybin.

vs control, $P \leq 0.01$) that decreased with silybin 50 $\mu\text{mol/L}$ (-38% with respect to steatotic cells, $P \leq 0.01$) (Figure 5B). Also NF- κ B activation showed a trend to an increase in steatotic cells and a significant reduction upon exposure to silybin 50 $\mu\text{mol/L}$ (-29% with respect to steatotic cells; $P \leq 0.05$) (Figure 5C).

DISCUSSION

The burden of nonalcoholic liver steatosis is rapidly increasing worldwide and is exposing the populations to the risk of liver-related complications^[1]. Ultimate therapeutic approaches are lacking so far, besides diet and physical exercise. The nutraceutical silybin has shown preliminary encouraging results either in clinical and animal studies^[39,40]. The present study provides a deeper characterization of some pathophysiologically relevant mechanisms of action of silybin administered as phytosome complex with vitamin E directly to fatty hepatocytes mimicking a steatosis condition *in vitro*. In this study, silybin disclosed direct anti-steatotic and anti-oxidant properties in steatotic cells, thus extending previous observations carried out by our group in studies with patients suffering with liver steatosis^[17], and in animal models fed a steatogenic diet^[41].

Accumulation of LDs within hepatocytes is the first step in the development of NAFLD^[42], while oxidative stress causing membrane lipid peroxidation and necro-inflammatory changes represents the second step leading to NASH^[43,44]. The exposure of hepatoma cells to exogenous FAs mimicked the first "hit" of NAFLD and led to TG accumulation within large cytosolic LDs that we observed by microscopic, fluorimetric and spectrophotometric analyses.

At the molecular level, TG accumulation in LDs seems to be sustained by the up-regulation of PPAR α that promotes FA transportation inside the cells, and by a larger up-regulation of PPAR γ , the lipogenic isoform that promotes TG synthesis from FAs. It has to be underlined that, although PPAR α is mostly known for its ability to induce FA oxidation, growing evidence points to its role in regulation of lipogenesis. In fact, PPAR α regulates many aspects of hepatic lipid metabolism including FA uptake through membranes, intracellular FA trafficking and oxidation, TG storage and lipolysis. In the present study, the changes in PPAR α mRNA expression are paralleled by those in the protein levels of SREBP-1c, that in fact is a PPAR α target gene^[45]. Moreover, PPAR α up-regulation can explain both the increase in lipophagy, TG secretion and in CPT-1 expression observed in steatotic cells. In fact, PPAR α typically promotes lipid mobilization from the cytosolic stores by stimulating both LD autophagy, lipid secretion and FA oxidation in mitochondria.

The activation of all these pathways represents an attempt of the cell to reduce excess fat accumulation, but are usually accompanied by increased ROS production. Indeed, the present data show that excess fat accumulation is accompanied by increased oxidative stress. To assess cellular oxidative stress, we measured: (1) content of ROS and hydrogen peroxide and activity of catalase, the main scavenger of H₂O₂; (2) lipid peroxidation, one of the most common indicators of free radical formation and a key indicator

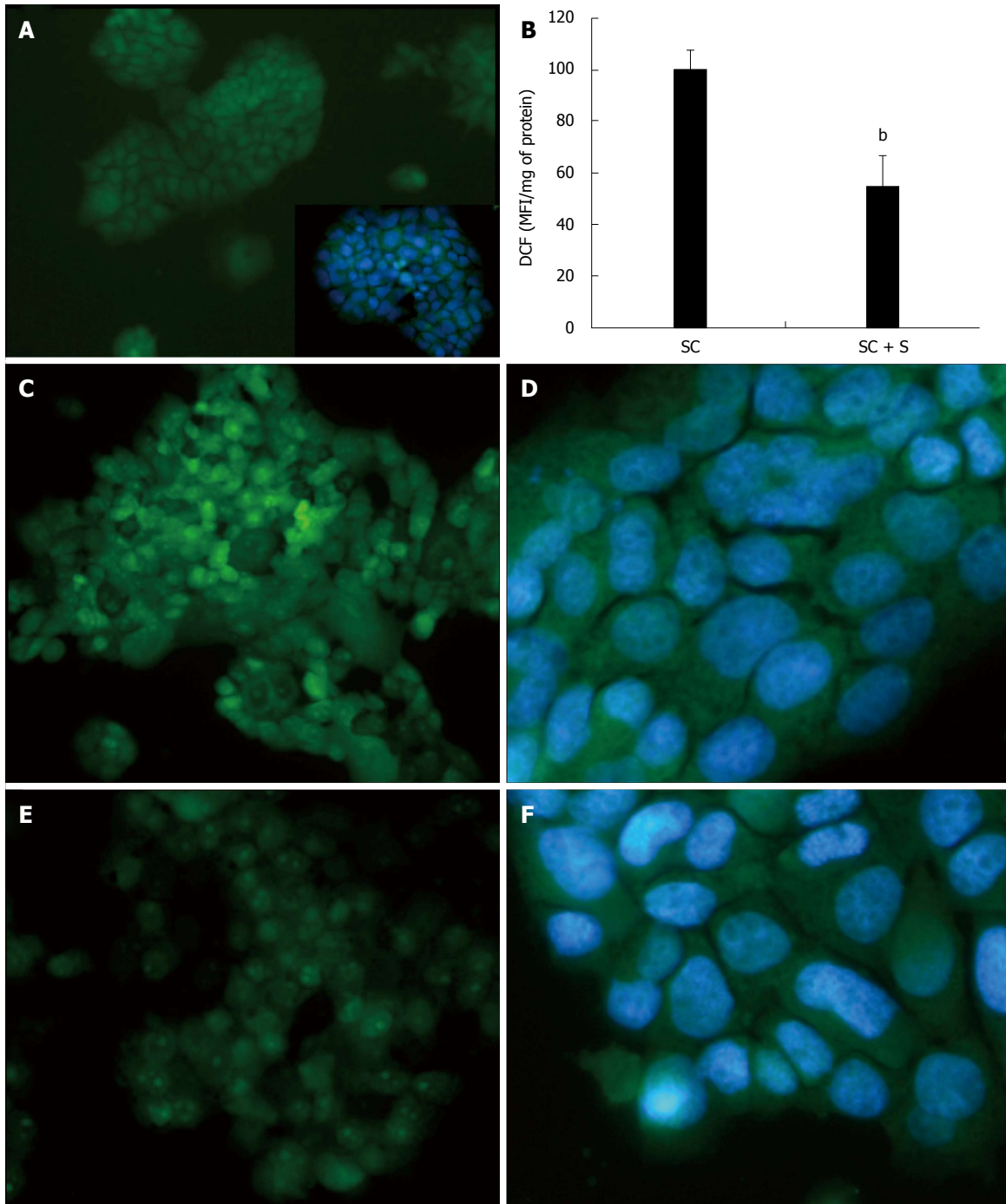


Figure 4 Effects of silybin on reactive oxygen species production. The intracellular level of reactive oxygen species (ROS), mainly hydrogen peroxide, was visualized *in situ* by optical microscopy in control (A and inset) and steatotic FaO cells incubated in the absence (C and D) or in the presence (E and F) of silybin 50 $\mu\text{mol/L}$. The ROS level were also quantified by spectrofluorimeter assay of DCF-stained cells (B) and data are expressed as percent mean fluorescence intensity (MFI) relative to steatotic cells and normalized for total proteins. DCF: 2'-7' dichlorofluorescein. ^b $P < 0.001$ SC vs SC + S

of oxidative stress; and (3) activation of NF- κ B, the master transcription factor in the control of molecular pathways related to oxidative stress. All these indices were increased in steatotic cells.

Incubation of steatotic cells with silybin 50 $\mu\text{mol/L}$ significantly reduces TG accumulation likely by promoting lipid catabolism pathways, as suggested by the up-regulation of PPAR α and PPAR δ , and by inhibiting lipogenic pathways, as suggested by the

down-regulation of PPAR γ . In fact, PPAR α and PPAR δ stimulation is known to promote hepatic fatty acid oxidation, and one of the strategies for improving lipid metabolism in metabolic diseases is to use a molecule which can simultaneously activate these two PPARs^[46], action that seems to be played by silybin. The stimulation of mitochondrial catabolism of FAs by silybin is also indicated by the marked up-regulation of CPT-1, which is the first component and rate-limiting

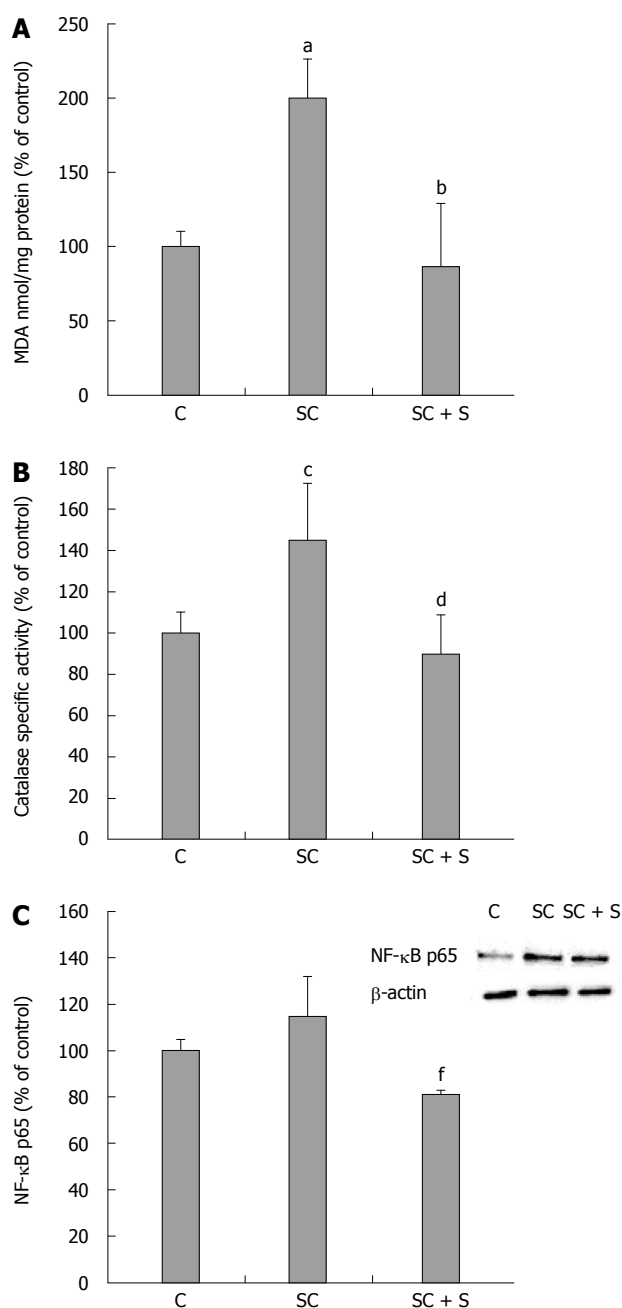


Figure 5 Effects of silybin on oxidative stress markers. In control and steatotic FaO cells incubated in the absence or in the presence of silybin 50 $\mu\text{mol/L}$ we assessed. A: The intracellular level of MDA (pmol MDA/mL \times mg of sample protein) quantified by TBARS assay data are expressed as percentage values with respect to controls and normalized for total proteins; B: Catalase specific activity (micromoles of decomposed H_2O_2 per min/mg of sample protein) evaluated by spectrophotometric assay, data are expressed as percentage values with respect to controls and normalized for total proteins. C: Densitometric analysis of nuclear NF- κ B/p65 evaluated by Western blotting; β -actin was the protein loading control used as housekeeping gene for normalization; data are expressed as percentage values with respect to controls. Values are mean \pm SD from at least three independent experiments. ANOVA followed by Tukey's test was used to assess the statistical significance between groups. Significant differences are denoted by symbols: ^a $P \leq 0.001$, ^c $P \leq 0.01$ C vs SC and ^b $P \leq 0.001$; ^d $P \leq 0.01$; ^f $P \leq 0.05$ SC vs silybin.

step of mitochondrial β -oxidation as it allows long chain fatty acids (LCFA) to enter the mitochondria. Many studies indeed indicate that the activity of

CPT1 determines the rate of LCFA oxidation and that over-expression of CPT1a in cultured cells increases fatty acid oxidation^[47,48]. On the other hand, silybin-dependent TG reduction occurred without a significant stimulation of lipophagic pathways and of TG release into the culture medium, as indicated by the unaltered levels of *Atg7* transcripts and extracellular TG content. It is noteworthy that this fact could be important for possible therapeutic applications.

In general, when TG storage inside LDs is inhibited, the extent of liver steatosis decreases, but liver damage might even increase due to excess of reactive free FAs produced by fat oxidation reactions. In fact, Teissier *et al.*^[49] found that PPAR agonists induce ROS production by stimulating NADPH oxidase activity. By contrast, in our study, the reduction in fat accumulation exerted by silybin in the steatotic hepatocytes is associated with the improvement of the oxidative unbalance caused by lipid excess as demonstrated by the reduction in lipid peroxidation (MDA levels, namely), catalase activity and NF- κ B activation.

Taken together, our results suggest that the antioxidant capacity of silybin effectively counteracts the possible damaging effects of a high rate of fat catabolism which is stimulated in the attempt to reduce excess FA accumulation. This step occurs by neutralizing the free radicals produced by fat oxidation reactions, thus rendering unnecessary the TG synthesis as a protection against toxic and proapoptotic effects of excess FAs^[50].

In conclusion, the present study points to the cellular modulatory and protective effect of silybin with respect to lipid accumulation, expression of lipid metabolism genes, TG secretion, and FA-driven oxidative stress. This study fits with prior studies investigating the beneficial effects of silybin on liver steatosis in animal models^[41], on inflammation and fibrosis in human hepatic stellate cells^[51] and in patients with NAFLD and metabolic disorders^[17]. Therefore silybin could represent a promising molecule for therapy of NAFLD and maybe of NASH. The prosecution of our work will be focused on testing the possible efficacy of silybin in NASH.

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COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of abnormal liver function tests in western countries, and evidences substantiate a strong association between NAFLD and metabolic abnormalities such as obesity, insulin resistance, and metabolic syndrome. Silymarin, the standardized extract from milk thistle (*Silybum marianum*), and its major active compound

silybin, have been used for a long time as a hepatoprotective agents for the treatment of acute and chronic liver diseases. However, the exact mechanisms of actions of this compound are still not completely clear.

Research frontiers

The identification of compounds which could improve the oxidative stress condition and counteract the liver damages may represent a useful instrument for the treatment of NAFLD to prevent its progression. This research field represents one of the most important challenge of the next few years for the scientific society.

Innovations and breakthroughs

Previous clinical and animal studies on silybin have found encouraging results showing its ability to improve liver disorders. This study demonstrates that silybin as phytosome complex with vitamin E favorably affects lipid and radical homeostasis through a direct action on hepatic cells. To this aim, the authors used an *in vitro* model of NAFLD induced by the exposure of hepatoma FaO cells to exogenous fatty acids. The intracellular triglyceride (TG) accumulation is mediated by increased levels of peroxisome proliferator-activated receptors (PPARs) and sterol regulatory element-binding protein-1c (SREBP-1c). The lipid imbalance is associated with higher production of reactive oxygen species (ROS) leading to increased lipid peroxidation, stimulation of catalase activity and activation of nuclear factor kappa-B (NF- κ B). The use of silybin significantly reduces TG accumulation likely by promoting lipid catabolism and by inhibiting lipogenic pathways, as suggested by the changes in carnitine palmitoyltransferase 1 (CPT-1), PPAR and SREBP-1c levels. The reduction in fat accumulation exerted by silybin in the steatotic cells is associated with the improvement of the oxidative imbalance caused by lipid excess as demonstrated by the reduction in ROS content, lipid peroxidation, catalase activity and NF- κ B activation.

Applications

This study indicates that silybin plays direct anti-steatotic and anti-oxidant effects in steatotic hepatocytes, thus supporting a potentially therapeutic use of this compound for preventing and or improving NAFLD progression.

Terminology

NAFLD refers to a group of conditions where there is accumulation of excess fat in the liver of people who drink little or no alcohol. Steatosis consist of excess content of triglycerides in hepatocytes. NAFLD definition encompasses a large spectrum of liver abnormalities which range from the simple steatosis, to nonalcoholic steatohepatitis, till to cirrhosis and hepatocellular carcinoma. Silybin is the major active constituent of silymarin, a standardized extract of the milk thistle seeds, containing a mixture of flavonolignans consisting of silibinin, isosilibinin, silicristin, silidianin and others.

Peer-review

The manuscripts describes that ability of silybin to counteract lipid excess and oxidative stress in cultured steatotic hepatic cells. This study presents interesting findings on the therapeutic effect of silybin, a flavolignan molecule of increasing interest for its beneficial effects. Both the problems and objectives of the manuscript are appropriated.

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Basic Study

Increased duodenal expression of miR-146a and -155 in pediatric Crohn's disease

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Abstract

AIM: To evaluate the role of microRNA (miR)-146a, -155 and -122 in the duodenal mucosa of pediatric patients with Crohn's disease (CD) and the effect of transforming growth factor- β (TGF- β) on these miRs in duodenal epithelial and fibroblast cells.

METHODS: Formalin-fixed, paraffin-embedded biopsies derived from the macroscopically inflamed (CD inflamed: $n = 10$) and intact (CD intact: $n = 10$) duodenal mucosa of pediatric CD patients and control children (C: $n = 10$) were examined. Expression of miR-146a, -155 and -122 was determined by real-time polymerase-chain reaction (PCR). The expression of the above miRs was investigated in recombinant human TGF- β (1 nmol/L, 24 h) or vehicle treated small intestinal epithelial cells (CCL-241) and primary duodenal fibroblast cells derived from healthy children as well.

RESULTS: Expression of miR-146a was significantly higher in the inflamed duodenal mucosa compared to the intact duodenal mucosa of children with CD (CD inflamed: 3.21 ± 0.50 vs CD intact: 0.62 ± 0.26 , $P \leq 0.01$) and to the control group (CD inflamed: 3.21 ± 0.50 vs C: 1.00 ± 0.33 , $P \leq 0.05$). The expression of miR-155 was significantly increased in the inflamed region of the duodenum compared to the control group (CD inflamed: 4.87 ± 1.02 vs Control: 1.00 ± 0.40 , $P \leq 0.001$). The expression of miR-122 was unchanged in the inflamed or intact mucosa of CD patients compared to controls. TGF- β treatment significantly decreased the expression of miR-155 in small intestinal epithelial cells (TGF- β : 0.7 ± 0.083 vs Control: 1 ± 0.09 , $P \leq 0.05$) and also the expression of miR-146a (TGF- β : 0.67 ± 0.04 vs Control: 1 ± 0.15 , $P \leq 0.01$) and miR-155 (TGF- β : 0.72 ± 0.09 vs Control: 1 ± 0.06 , $P \leq 0.05$) in primary duodenal fibroblasts compared to corresponding vehicle treated controls. TGF- β treatment did not influence the expression of miR-122.

CONCLUSION: The elevated expression of miR-146a and -155 in the inflamed duodenal mucosa of CD patients suggests the role of these miRs in the pathomechanism of inflammatory bowel disease. Anti-inflammatory TGF- β plays an important role in the regulation of the expression of these miRs.

Key words: Inflammatory bowel disease; Crohn's disease; Pediatric; MicroRNAs; Transforming growth factor- β

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Core tip: Recent evidence suggests that besides the genetic basis, epigenetic factors including microRNAs (miRs) also act as potent inflammatory modulators during the pathogenesis of inflammatory bowel disease. MiR expression in the upper-gastrointestinal tract of pediatric patients with Crohn's disease (CD) has not yet been analyzed. Moreover, the relation of transforming growth factor- β , playing a prominent role in the pathomechanism of CD, to miRs in this setting is also unknown. The description of precise miR patterns specific for the different segments of the gastrointestinal tract may contribute to the introduction of novel diagnostic markers and to the identification of potential therapeutic targets.

Szűcs D, Béres NJ, Rokony R, Boros K, Borka K, Kiss Z, Arató A, Szabó AJ, Vannay Á, Sziksz E, Bereczki C, Veres G. Increased duodenal expression of miR-146a and -155 in pediatric Crohn's disease. *World J Gastroenterol* 2016; 22(26): 6027-6035 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i26/6027.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i26.6027>

INTRODUCTION

Crohn's disease (CD) is a chronic immune-mediated disorder, which is frequently characterized by the appearance of lesions in the upper gastrointestinal (GI) tract, primarily in pediatric patients^[1,2]. In adults, upper GI lesions in the cardiac area with bamboo joint-like appearance^[3] are regarded to be significant risk factors for the progression of the disease from the inflammatory to stenotic or penetration form of CD^[4].

In the pathogenesis of CD, epigenetic factors including microRNAs (miRs) have come into focus as potent modulators of the progression of the disease. An increasing number of studies investigates the role of these short single-stranded RNAs in inflammatory bowel disease (IBD)^[5-11]; however, the expression profile of miRs in the upper GI region of CD patients is completely unknown.

The aim of the present study was to investigate the mucosal expression of miR-146a, -155 and -122 in the upper GI tract of children with CD, and to examine the effect of the anti-inflammatory transforming growth factor (TGF)- β on the expression of the investigated miRs. These miRs affect a number of key biological functions involved in the pathomechanism of CD, including inflammatory response, intracellular signaling cascades and response to the presence of bacteria^[5].

However, upper GI tract endoscopy is an important tool to diagnose CD in pediatric patients, recently there have been no specific markers to definitely distinguish it from other GI diseases with erosions, ulcerations [*Helicobacter pylori* (*H. pylori*) infection, peptic ulcer caused by an infection or medication, or eosinophil gastritis], and disorders with granulomas (sarcoidosis, *Mycobacterium tuberculosis*). Therefore, we suppose that our results may contribute to the identification of potential novel biomarkers or therapeutic targets of CD.

MATERIALS AND METHODS

Patients

CD was diagnosed according to the Porto criteria^[12,13]. The disease activity score was assessed regarding the Pediatric CD Activity Index (PCDAI). Control children were referred to the outpatient clinic due to recurrent abdominal pain and GI symptoms to exclude organic diseases. Esophago-gastro-duodenoscopy was part of their diagnostic procedure showing normal macroscopic appearance and histology. Duodenal biopsy samples were taken from different patients, macroscopically inflamed (CD inflamed: $n = 10$) and non-inflamed (CD intact: $n = 10$) regions of the duodenal mucosa from children with CD and controls (C: $n = 10$). Biopsies were immediately fixed in buffered formaldehyde and embedded into paraffin (PF). Clinical characteristics

Table 1 Clinical characteristics of patients

| | Control | CD intact | CD inflamed |
|--------------------------|---------------|----------------------------|---------------------------|
| <i>n</i> | 10 | 10 | 10 |
| Age | 8.75 ± 2.36 | 12.4 ± 1.52 | 12.11 ± 1.63 |
| Gender | 2f/8m | 5f/5m | 2f/8m |
| BMI (kg/m ²) | 20.07 ± 2.64 | 14.83 ± 0.8 | 18.64 ± 1.81 |
| PCDAI | 0 | 21.94 ± 4.37 ^c | 16.11 ± 3.51 ^b |
| Iron (μmol/L) | 15.83 ± 2.53 | 4.89 ± 0.84 ^b | 8.33 ± 2.61 ^a |
| TIBC (μmol/L) | 60.4 ± 2.71 | 48.5 ± 2.64 | 59.71 ± 8.70 |
| Albumin (g/L) | 47.33 ± 1.31 | 38.67 ± 1.62 | 48.4 ± 5.56 |
| Hemoglobin (g/L) | 132.8 ± 6.72 | 113.1 ± 4.02 ^a | 124.4 ± 5.93 |
| Hematocrit (%) | 0.38 ± 0.02 | 0.344 ± 0.01 | 0.37 ± 0.02 |
| Platelet count (Giga/L) | 365.5 ± 30.29 | 517.1 ± 34.62 ^a | 416.7 ± 63.77 |

^a*P* ≤ 0.05, ^b*P* ≤ 0.01, ^c*P* ≤ 0.001 *vs* Control. BMI: Body mass index; CD: Crohn's disease; PCDAI: Pediatric Crohn's disease activity index; TIBC: Total iron binding capacity.

of patients with CD and controls are shown in Table 1. Written informed consent was obtained from the parents prior to the procedure, and the study was approved by the Semmelweis University Regional and Institutional Research Ethics Committee (TUEB No.: 10408/2012).

TGF-β treatment of duodenal epithelial cells and primary fibroblasts

Normal small intestinal epithelial cells (CCL-241, American Type Culture Collection, Manassas, VA, United States) were grown in HybriCare medium (American Type Culture Collection, Manassas, VA, United States) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, United States), 30 ng/mL epidermal growth factor (R&D Systems, Minneapolis, MN, United States), and 1% Penicillin and Streptomycin mixture (Sigma-Aldrich Co., St. Louis, MO, United States). Epithelial cells were grown under standard cell culture conditions (37 °C, humidified, 5% CO₂/95% air environment).

Based on the previously described method, primary duodenal fibroblast cells were freshly isolated^[14] from the duodenal mucosa of healthy children. Briefly the biopsies were washed in phosphate-buffered saline (PBS) and homogenized in 1 mg/mL collagenase content PBS (Sigma-Aldrich Co., St. Louis, MO, United States). Cells were grown in Dulbecco's Modified Eagle Medium supplemented with 1% FBS (Invitrogen, Carlsbad, CA, United States) and 1% Penicillin and Streptomycin mixture. Cells were grown under standard cell culture conditions (37 °C, humidified, 5% CO₂/95% air environment) until a confluent monolayer was obtained. During culturing, the unattached cells were removed after every 24 h culture period.

Epithelial and primary fibroblast cells were seeded into 6-well plates at a density of 5 × 10⁵ cells/well and treated for 24 h with recombinant human TGF-β (R&D Systems, Minneapolis, MN, United States) at a concentration of 1 nmol/L or vehicle only for control cells.

RNA isolation

Total RNA was isolated from formalin-fixed, paraffin-embedded biopsies using RNeasy Minikit (Qiagen, Düsseldorf, Germany) after removing paraffin from the samples according to the instructions of the manufacturer. All contaminants were efficiently washed away, DNase was used to remove DNA from the samples using on-column DNase treatment. Concentrated RNA was filtrated using RNeasy MinElute spin columns (Qiagen, Düsseldorf, Germany). RNA was eluted in 30 μL water.

Total RNA of the intestinal epithelial and fibroblast cells was isolated by the Quick-RNA MiniPrep isolation kit (Zymo Research, Irvine, CA, United States) according to the instructions of the manufacturer. RNA Lysis Buffer purified the RNA using Zymo-Spin™ Columns. All contaminants were washed away (RNA Prep and Wash Buffer) and DNase was used to remove the DNA from the samples. RNA was eluted in 30 μL water and used further immediately.

Reverse transcription and real-time polymerase-chain-reaction

The total RNA was reversely transcribed by the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems - ABI, Foster City, CA, United States). TaqMan MicroRNA Assay (Applied Biosystems - ABI, Foster City, CA, United States) was used to quantify individual microRNA levels with real-time Reverse transcription and real-time polymerase-chain-reaction (RT-PCR) on a LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland). Reactions were performed in triplicates. Relative expression of miRs was determined by the 2ΔCq method using U6 as an internal control.

Statistical analysis

Statistical analysis was performed by Graphpad statistical software package (Graphpad Software, La Jolla, CA, United States). Normality was tested by the Shapiro-Wilk test. Analysis was based on the Mann-Whitney *U*-test, Kruskal-Wallis-test, Analysis of variance (ANOVA) and Dunn's Post-Hoc test, and *P* ≤ 0.05 was considered as statistically significant. Data were presented as mean ± SE.

RESULTS

Expression of miR-146a in the duodenal mucosa of pediatric patients with CD

The expression of miR-146a was significantly higher in the inflamed duodenal mucosa of children with CD compared to the intact mucosa (CD inflamed: 3.21 ± 0.50 *vs* CD intact: 0.62 ± 0.26, *P* ≤ 0.01) and controls (CD inflamed: 3.21 ± 0.50 *vs* Control: 1.00 ± 0.33, *P* ≤ 0.05). There was no significant difference between the uninflamed group and the control one (CD intact

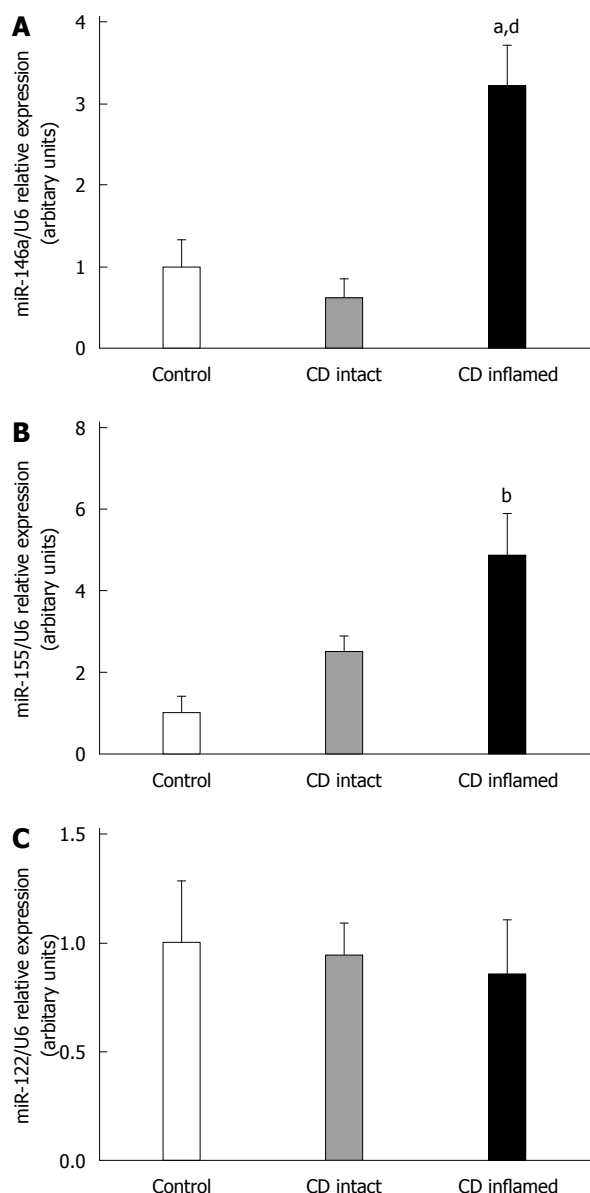


Figure 1 Expression of microRNA-146a (A), microRNA-155 (B) and microRNA-122 (C) in the duodenal mucosa of pediatric patients with Crohn's disease. Expression of microRNA (miR)-146a was significantly higher in the inflamed duodenal mucosa of children with Crohn's disease (CD) compared to the intact mucosa and controls. miR-155 showed significantly elevated expression in the inflamed region of the duodenum compared to the control group. There was no significant difference in the expression of miR-122 between the groups. ^a $P \leq 0.05$ vs Control; ^b $P \leq 0.001$ vs Control; ^d $P \leq 0.01$ vs CD intact.

vs Control: $P = \text{N.S.}$) (Figure 1A).

Expression of miR-155 in the duodenal mucosa of pediatric patients with CD

miR-155 showed significantly elevated expression in the inflamed region of the duodenal mucosa of CD patients compared to the control group (CD inflamed: 4.87 ± 1.02 vs Control: 1.00 ± 0.40 , $P \leq 0.001$). There was no significant difference between the uninfamed group and the control one (CD intact: 2.50

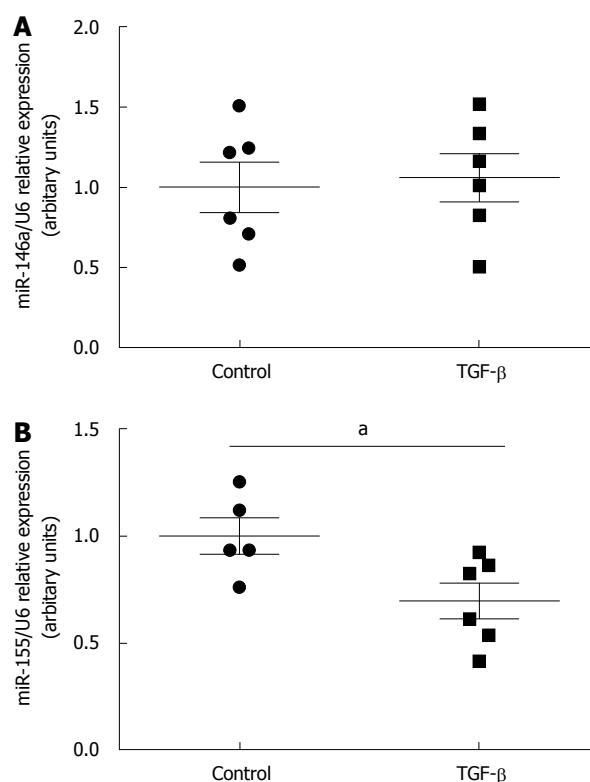


Figure 2 Effect of transforming growth factor- β on the expression of microRNA-146a (A), and microRNA-155 (B) in duodenal epithelial cells. Transforming growth factor (TGF)- β did not have any effect on the expression of microRNA (miR)-146a. TGF- β significantly decreased the expression of miR-155. There was no miR-122 expression detected, ^a $P \leq 0.05$ vs Control.

± 0.38 vs Control: 1.00 ± 0.40 , $P = \text{N.S.}$) (Figure 1B).

Expression of miR-122 in the duodenal mucosa of pediatric patients with CD

There was no significant difference in the expression of miR-122 between the CD and control groups (CD inflamed: 0.86 ± 0.25 , CD intact: 0.96 ± 0.14 , Control: 1.00 ± 0.28 , $P = \text{N.S.}$) (Figure 1C).

Expression of miR-146a, -155 and -122 in TGF- β treated duodenal epithelial cells

TGF- β had no effect on the expression of miR-146a ($P = \text{N.S.}$, Figure 2A); however, it decreased significantly the expression of miR-155 in CCL-241 small intestinal epithelial cells (TGF- β : 0.7 ± 0.083 vs Control: 1 ± 0.09 , $P \leq 0.05$) (Figure 2B). No miR-122 was detected in the small intestinal epithelial cells.

Expression of miR-146a, -155 and -122 in TGF- β treated duodenal fibroblasts

TGF- β significantly decreased the expression of miR-146a (TGF- β : 0.67 ± 0.04 vs Control: 1 ± 0.15 , $P \leq 0.01$) (Figure 3A) and miR-155 (TGF- β : 0.72 ± 0.09 vs Control: 1 ± 0.06 , $P \leq 0.05$) (Figure 3B) in duodenal fibroblasts compared to vehicle treated control cells. There was no difference in the expression of miR-122

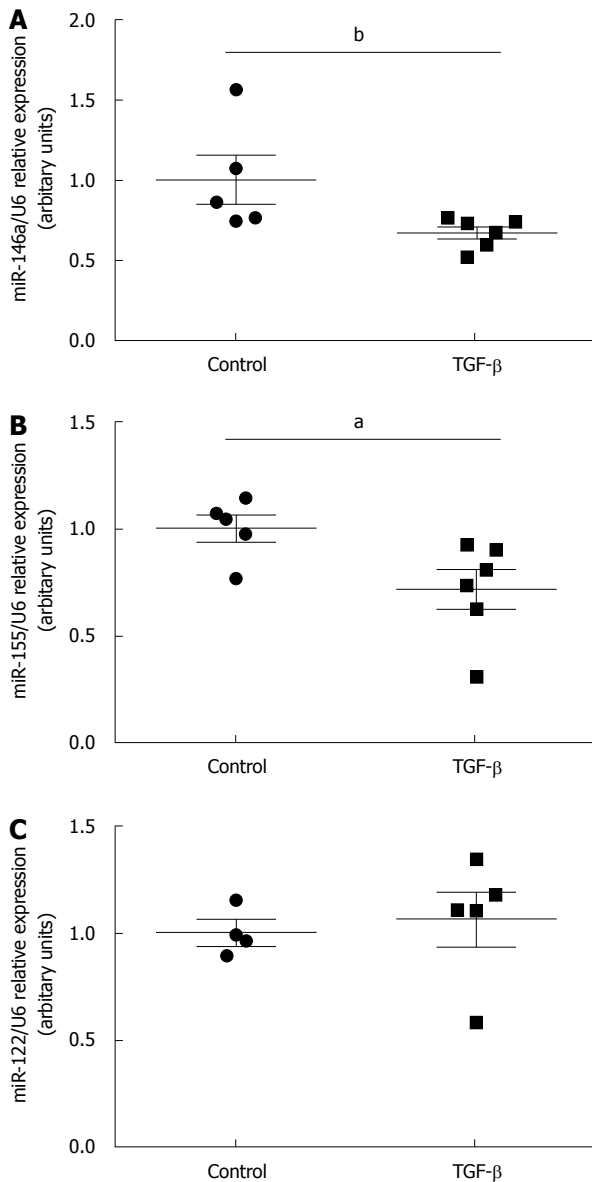


Figure 3 Effect of transforming growth factor- β on the expression of microRNA-146a (A), microRNA-155 (B), and microRNA-122 (C) in duodenal fibroblasts. Transforming growth factor (TGF)- β significantly decreased the expression of microRNA (miR)-146a and miR-155. TGF- β had no effect on the expression of miR-122, ^a $P \leq 0.05$ vs Control, ^b $P \leq 0.01$ vs Control.

when TGF- β was administered compared to the control group ($P = \text{N.S.}$, Figure 3C).

DISCUSSION

In the present study, we demonstrated the elevated expression of miR-146a and -155 in the macroscopically inflamed duodenal mucosa of newly diagnosed, treatment-naïve pediatric patients with CD compared to the control group for the first time. These results are in accordance with our previous observations demonstrating the increased expression of miR-146a and -155 in the inflamed colonic region of pediatric IBD patients. However, in contrary to our earlier observations related to the colon, we found unchanged

expression of miR-122 in the duodenal mucosa of children with CD^[5].

According to the criteria of the "Porto" IBD Working Group of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), upper GI tract endoscopy is recommended to be performed to identify lesions in all children irrespective of presence or absence of upper GI symptoms^[15]. Based on the Hungarian Pediatric IBD Registry Group (HUPIR), upper GI tract abnormalities were found in 64% and 40% of children with CD and UC, respectively^[16]. Characteristic findings (ulcers, erosions, aphthous lesions, and granulomas) were noted in about one third of children with CD; moreover, upper GI tract endoscopy was advantageous in establishing the final diagnosis in 9% of children with CD (diagnostic yield)^[16]. However, to the best of our knowledge, there is no specific marker to clearly diagnose CD in the upper GI tract and to distinguish it from *H. pylori* infection, eosinophilic enteropathy or drug-induced lesions. Moreover, *H. pylori*-negative chronic active gastritis appears frequently in adult patients with CD, which is hard to differentiate from CD lesions^[17]. Therefore, the aim of our present study was to establish a duodenum-specific miR pattern in pediatric patients with CD, which could facilitate the deeper understanding of the pathomechanism of IBD, and it may serve as a diagnostic tool in the future.

Recently miR-146a, -155 and -122 have come into focus as potential regulators of the inflammatory response, intracellular signaling cascades, regulation of cytokine production and response to bacteria, all of which play an important role in IBD. The main findings of the previous studies regarding to the expression of miR-146a, -155 and -122 in the GI tract of adult and pediatric IBD patients also confirm it. Based on these results and our present findings, we can conclude that independently of the localization of the intestinal inflammatory regions (colon, rectum, or duodenum), the expression of miR-146a and -155 was elevated in patients with CD suggesting that these miRs are rather inflammation- than region-specific^[5,8,18-26]. Both *in vivo* and *in vitro* studies confirm that miR-146a and -155 have opposite effects during inflammation. While miR-155 is a promoter of inflammation, miR-146a is a mediator of immune suppressive responses^[27,28].

In contrary to miR-146a and -155, different expression pattern of miR-122 has been demonstrated throughout the GI tract. Previously we observed elevated expression of miR-122 in the intact colonic region of pediatric CD patients^[5], but in the present study we found no difference in the duodenal miR-122 expression between CD patients and the control ones. Chen *et al.*^[29] have found that miR-122 may decrease intestinal injury by suppressing the NOD2-induced NF- κ B signaling pathway. On the contrary, overexpression of miR-122 in enterocytes have resulted in the mRNA degradation of occludin leading to disturbed tight-

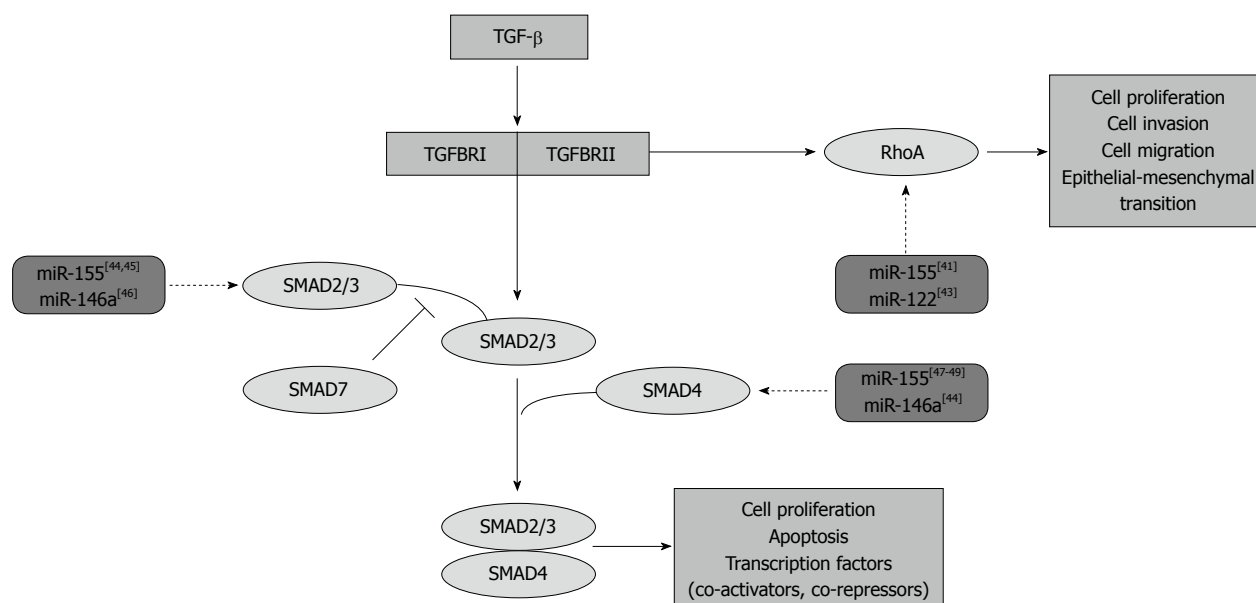


Figure 4 Schematic representation of the target interactions between transforming growth factor- β - microRNA-146a, -155 and -122. Transforming growth factor (TGF)- β is considered to be a major anti-inflammatory cytokine playing an important role in the pathogenesis of inflammatory bowel disease. MicroRNA (miR)-146a, -155 and -122 act as possible regulators of the TGF- β signal transduction, with capacity to induce apoptosis, cell migration, invasion and proliferation. Moreover, TGF- β induces and represses the transcription of various genes. Data shown in the figure are based on MiRTarBase Database. SMAD: Mothers against decapentaplegic homolog; RhoA: Ras homolog gene family, member A.

junction integrity and increased intestinal permeability, which are important processes in the pathogenesis of IBD^[30,31].

Inflammatory factors have been shown to modulate the expression of miRs, but the potential regulators of miR-146a, -155 and -122 in IBD are largely unknown. One of the known key modulators of inflammatory responses is anti-inflammatory TGF- β ^[32-34].

In case of IBD, increased expression of TGF- β was demonstrated in the inflamed intestinal regions. However, due to the reduced level of phosphorylated mothers against decapentaplegic homolog (SMAD)3 and diminished complex formation with SMAD4, the TGF- β -mediated signaling is insufficient in IBD patients^[35].

Moreover, elevated level of SMAD7, the known endogenous inhibitor of TGF- β signaling, contributes to the enhanced production of pro-inflammatory cytokines resulting in the maintenance of inflammation in IBD^[36,37]. This disadvantageous effect of SMAD7 in IBD is proved by its inhibition in a human phase II study. In fact, the oral administration of SMAD7 targeting antisense oligonucleotides has led to clinical remission in 60% of adult CD patients^[38]. Moreover, Rho proteins, which are also connected to the TGF- β signaling pathway are involved in the regulation of IBD. Previous studies have reported Ras homolog gene family member A (RhoA) protein activation in CD patients. RhoA is involved in inflammation through the activation of the NF- κ B, IL-1 β and the TNF- α pathway, regulation of cell adhesion, migration, phagocytosis, and proliferation^[39,40] (Figure 4).

Based on these key effects of TGF- β in the patho-

mechanism of IBD, we also aimed to investigate the possible link between TGF- β and miR-146a, -155 and -122. Following TGF- β treatment, we observed downregulation of miR-155 in both small intestinal epithelial and primary duodenal fibroblast cells and reduced expression of miR-146a in duodenal fibroblasts. However, TGF- β had no effect on the expression of miR-122 either in epithelial or in fibroblast cells.

Previous studies have confirmed the effect of these miRs on the TGF- β -mediated signaling pathway^[41-49] (Figure 4). In chondrocytes, fetal femur derived skeletal stem cells and gastric cancer cells miR-146a negatively regulates the expression of SMAD 2/3 and SMAD4, while in monocyte, cervical and prostate cancer cells miR-155 negatively modulates SMAD2/3, the key mediators of TGF- β -induced effects^[46,47,49,50]. Moreover, the ectopic expression of miR-155 and -122 has significantly reduced the expression of RhoA indicating the role of these miRs in the TGF- β -induced epithelial-mesenchymal transition, cell migration and invasion, as well^[41,43].

In conclusion, increased expression of miR-146a and -155 in the inflamed intestinal mucosa suggests their involvement in the pathomechanism of CD as inflammation-specific markers. Our results suggest that the expression of miR-146a and -155 is independent of and that of miR-122 is dependent on the localization of CD. Moreover, anti-inflammatory TGF- β is a negative regulator of miR-146a and -155 in small intestine epithelial and primary duodenal fibroblast cells. Our recent data have provided a baseline to explore the possible role of these miRs as diagnostic markers or

their potential as therapeutic targets.

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COMMENTS

Background

Crohn's disease (CD) is a chronic immune-mediated disorder frequently characterized by lesions of the upper gastrointestinal (GI) tract, primarily in pediatric patients. Recently, epigenetic factors including microRNAs (miRs) have come into focus as potent modulators of the progression of the disease. An increasing number of studies investigate the role of these short single-stranded RNAs in inflammatory bowel disease (IBD); however, the expression profile of miRs in the upper GI region of pediatric CD patients is completely unknown.

Research frontiers

In the previous study, the authors observed increased expression of miR-146a, -155 and -122 in the inflamed colonic region of pediatric IBD patients.

Innovations and breakthroughs

This is the first study investigating the mucosal expression of miR-146a, -155 and -122 in the upper GI tract of children with CD and examining the effect of anti-inflammatory transforming growth factor (TGF)- β on their expression of small intestinal epithelial and primary duodenal fibroblast cells.

Applications

Increased expression of miR-146a and -155 in the inflamed intestinal mucosa of pediatric patients with CD suggests their involvement in the pathomechanism of CD as inflammation-specific markers. Anti-inflammatory TGF- β is a negative regulator of miR-146a and -155 in the small intestinal epithelial and primary duodenal fibroblast cells. The recent data provide a baseline to explore the possible use of these miRs as diagnostic markers or their potential role as therapeutic targets.

Terminology

MiRs are 19-24 nucleotide-long single-stranded RNAs involved in the regulation of gene expression at transcriptional and posttranscriptional level. MiRs play a determining regulatory role in innate and adaptive immune processes.

Peer-review

The authors demonstrated an interesting study based on their previous findings connected to the expression of miR-146a, -155 and -122 in the colonic mucosa of pediatric inflammatory bowel disease. It would be interesting to measure the effect of the pro-inflammatory tumor-necrosis-factor- α on the small intestine epithelial and primary duodenal fibroblast cells as a future plan.

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Basic Study

Umbilical cord-derived mesenchymal stem cells alleviate liver fibrosis in rats

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Author contributions: Chai NL performed the majority of experiments and analyzed the data; Zhang XB, Chen SW and Fan KX participated equally in the treatment of animals and completed the paper writing task together; Linghu EQ designed and coordinated the research.

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Institutional review board statement: Our research was performed under the approval of the Local Ethics Committee of Chinese PLA Medical Academy (Beijing China), with permit number 2012-32.

Institutional animal care and use committee statement: All of the procedures were performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health strictly. Appropriate measures were taken to minimize the animals' pain or discomfort.

Conflict-of-interest statement: We declare that we have no financial and personal relationships with other people or organizations that could inappropriately influence our work.

Data sharing statement: The technical appendix, statistical code and dataset are available from the first author Ning-Li Chai at csxlily@163.com. All the participants gave informed consent for data sharing.

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Abstract

AIM: To evaluate the efficacy of umbilical cord-derived mesenchymal stem cells (UC-MSCs) transplantation in the treatment of liver fibrosis.

METHODS: Cultured human UC-MSCs were isolated and transfused into rats with liver fibrosis induced by dimethylnitrosamine (DMN). The effects of UC-MSCs transfusion on liver fibrosis were then evaluated by histopathology; serum interleukin (IL)-4 and IL-10 levels were also measured. Furthermore, Kupffer cells (KCs) in fibrotic livers were isolated and cultured to analyze their phenotype. Moreover, UC-MSCs were co-cultured with KCs *in vitro* to assess the effects of UC-MSCs on KCs' phenotype, and IL-4 and IL-10 levels were measured in cell culture supernatants. Finally, UC-MSCs and KCs were cultured in the presence of IL-4 antibodies to block the effects of this cytokine, followed by phenotypical analysis of KCs.

RESULTS: UC-MSCs transfused into rats were recruited by the injured liver and alleviated liver fibrosis, increasing serum IL-4 and IL-10 levels. Interestingly, UC-MSCs promoted mobilization of KCs not only in fibrotic livers, but also *in vitro*. Co-culture of UC-MSCs with KCs resulted in increased production of IL-4 and IL-10. The addition of IL-4 antibodies into the co-culture system resulted in decreased KC mobilization.

CONCLUSION: UC-MSCs could increase IL-4 and promote mobilization of KCs both *in vitro* and *in vivo*, subsequently alleviating the liver fibrosis induced by DMN.

Key words: Liver fibrosis; Mesenchymal stem cells; Kupffer cells; Interleukin-4; Dimethylnitrosamine; DMN

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Core tip: Dysregulation of the M1/M2 macrophages phenotypic balance governs the pathogenesis of liver fibrosis. Flow cytometry, immunohistochemistry and liver function tests showed that umbilical cord-derived mesenchymal stem cells (UC-MSCs) could promote the mobilization of M1 Kupffer cells (KCs) into the M2 phenotype *in vivo* and *in vitro* thereby ameliorating liver inflammation and liver fibrosis. Thus, UC-MSC transfusion yielded promising results with regard to reversal of liver injury and alleviated liver fibrosis by promoting KC mobilization and hepatocyte differentiation. The application of UC-MSCs might provide a new tool for cell therapy of liver fibrosis.

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INTRODUCTION

Liver fibrosis is attributed to the excess deposition of collagen. It is usually caused by chronic liver injury, which triggers hepatocyte apoptosis, inflammatory cell recruitment, endothelial barrier damage, increased levels of transforming growth factor $\beta 1$ (TGF- $\beta 1$) and activated myofibroblast, which are responsible for scar tissue formation^[1]. Inflammation might be the most critical factor in the initiation and maintenance of liver fibrogenesis^[1]. When the liver is injured, the damaged epithelial and endothelial cells release inflammatory mediators, and the peripheral blood inflammatory cells are recruited to the affected liver, releasing fibrosis-related mediators such as TGF- $\beta 1$ and tumor necrosis factor- α (TNF- α), inducing the activation of hepatic stellate cells and as well as deposition of collagen. Anti-smooth muscle α -actin (α -SMA) is a marker of

activated hepatic stellate cells (HSCs), and HSCs play key roles in the pathogenesis of liver fibrosis. It is acknowledged that liver fibrosis can be effectively reversed^[1], and the promotion of the repair process is considered a therapeutic strategy for liver fibrosis.

Currently, stem cell therapy is considered a promising treatment for various liver diseases, with most studies yielding positive results^[2]. Mesenchymal stem cells (MSCs) are the most commonly used stem cells in transplantation. They are multipotent, non-hematopoietic progenitor cells that can differentiate into multiple lineages and have been applied in tissue regeneration and repair. Their hypo-immunogenicity and potential immunomodulatory capacity ensure that the MSCs have clinical value^[2]. Increasing evidence suggests that MSCs contribute to the direct production of new hepatocytes^[3,4]. Among MSCs, the umbilical cord-derived MSCs (UC-MSCs) possess an excellent proliferative potential, and their low immunogenicity and ease of preparation make them a good choice for use in future clinical studies^[5]. Previous studies have shown that UC-MSCs are a well-tolerated therapy. They have the potential to improve the liver function and reduce ascites and mortality, especially in hepatitis B virus patients with decompensated liver cirrhosis^[6] and liver failure^[7]. Although the effects of UC-MSCs on liver fibrosis had been confirmed in many studies, the detailed mechanism remains unclear.

TGF- $\beta 1$ is a potent fibrogenic cytokine, playing an important role in the activation of fibrogenic myofibroblasts. In fibrosis, its major source is the Kupffer cells (KCs; liver resident macrophages)^[8]. Many clinical and experimental data have indicated that the activation of KCs is the key step in the initiation of liver injury^[9-11]. Macrophages are divided into two major cell subpopulations: classically activated proinflammatory M1 macrophages and alternatively activated anti-inflammatory or wound repair M2 macrophages. The M1 type is induced by interferon γ (IFN γ), TLR-4 ligands and bacterial infection, while the M2 type is mostly induced by Interleukin-4 (IL-4), IL-10 or TGF- β ^[12]. Several studies^[13-15] have demonstrated that when the liver is injured, these two functionally distinct macrophage types will be recruited to it. During the injury phase, pro-fibrogenic macrophages (M1) promote myofibroblast proliferation and apoptosis. In contrast, during the injury repair phase, the M2 macrophages predominate and mediate matrix degradation^[16]. Some papers have confirmed that M2 macrophages are present during the injury repair phase when the levels of pro-fibrogenic and inflammatory mediators are decreasing^[13]. Therefore, the disequilibrium between M1 and M2 macrophages appears to be the major pathogenesis that induces liver fibrosis. Strategies for restraining M1 macrophage mobilization or encouraging the M2 macrophage phenotype might prevent liver injury and thus alleviate liver fibrosis.

The goal of our study was to evaluate the efficacy of UC-MSCs transplantation to treat liver fibrosis in

rats. Furthermore, because activation of KCs is the key step in the initiation of liver injury, we were also interested in the influence of UC-MSCs transplantation on the mobilization of KCs and its mechanism.

MATERIALS AND METHODS

Isolation, culture and identification of human UC-MSCs

Research protocols that involved human participants were reviewed and approved by the Local Ethics Committee of Chinese PLA Medical Academy (Beijing, China). Written informed consent was provided by each participant in advance. Human umbilical cord samples were obtained from umbilical veins immediately after cesarean section, with the mother's consent, at the Chinese PLA Medical Academy of the PLA general hospital. We mixed the umbilical cord samples with HetaSep solution (Stemcell Technologies, Vancouver, BC, Canada) at a ratio of 5:1 and incubated the mixture at room temperature to deplete erythrocytes. We then collected the supernatant carefully and used Ficoll density-gradient centrifugation at $398 \times g$ for 20 min to obtain the mononuclear cells. We washed the cells once or twice in phosphate-buffered saline (PBS) and seeded them into plates at a density of 2×10^5 to 2×10^6 cells/cm². We incubated these cells in a humidified atmosphere containing 5% CO₂ at 37 °C. The cells were then resuspended in 10 mL MSC medium (Alpha Modified Eagles Medium, Invitrogen, Carlsbad, CA, United States), supplemented with 10% fetal bovine serum (FBS; Invitrogen), 10% horse serum (Invitrogen), and 5 mg/mL streptomycin and 5 IU/mL penicillin (Sigma, St. Louis, MO, United States). We collected the mixture in a 15 mL Falcon tube and centrifuged it at $238 \times g$ for seven minutes at 4 °C. The cells were then counted and added to a 75 cm² flask containing 15 mL of MSC medium. The adherent cells formed colonies and grew rapidly, exhibiting a spindle-shaped morphology. When the UC-MSCs reached 85% confluence, they were passaged, and cells at the fourth passage were used for transfusion into rats. Before transfusion, UC-MSCs were subjected to quality control, including the detection of CD14, CD19, CD34, CD44, CD45, CD73 and CD105 by flow cytometry analysis, and bacteriological testing.

Standard osteogenic, adipogenic and chondrogenic assays were used to assess their differentiation potential. Osteogenic differentiation of confluent UC-MSCs monolayers obtained as described above was induced using 100 nmol/L dexamethasone, 0.05 mmol/L L-ascorbic acid-2-phosphate and 10 mmol/L β -glycerophosphate (all from Sigma). Alizarin red staining using a Sigma kit was performed to observe calcium deposition in the cultures. Adipogenic differentiation was induced in DMEM/10% FBS supplemented with 0.5 mmol/L isobutylmethylxanthine (Sigma), 60 mol/L indomethacin (ICN, Basingstoke, United Kingdom) and 0.5 mmol/L hydrocortisone

(Sigma). Accumulation of lipid vacuoles was visualized using 0.5% Oil Red-O. For chondrogenic differentiation, cells (2.5×10^5) were placed in serum-free medium consisting of high-glucose DMEM, 100 μ g/mL sodium pyruvate, 40 μ g/mL proline, 50 μ g/mL L-ascorbic acid-2-phosphate, 1 mg/mL BSA, $1 \times$ insulin-transferrin-selenium plus, 100 nmol/L dexamethasone (all from Sigma) and 10 ng/mL transforming growth factor- β 3 (TGF β 3; R&D Systems, Abingdon, United Kingdom). Cell culture media were replaced every other day. Micromasses were harvested at week 3, and frozen sections (5- μ m thick) were prepared.

Flow cytometry analysis

Low- and high-passage MSCs were analyzed by flow cytometry. Briefly, 2×10^5 to 2×10^6 cells were plated into each well of a round-bottom 96-well plate. After washing with 0.1 mol/L PBS containing 1% bovine serum albumin (Sigma) and 0.1% azide (Sigma), cells were resuspended in 30 μ L of primary antibodies against CD14, CD19, CD34, CD44, CD45, CD73, and CD105 (Santa Cruz Biotechnologies, Dallas, Texas, United States) for one hour at 4 °C, respectively. The cells were then rinsed twice and incubated in secondary antibody conjugated to AlexaFluor488 (Invitrogen) for one hour at 4 °C. Then, cells were rinsed twice, fixed using 4% paraformaldehyde for ten minutes on ice, and stored at 4 °C until analysis on a flow cytometer (BD Biosciences, San Diego, CA, United States).

Animal model of liver fibrosis and UC-MSCs transplantation

All of the Sprague-Dawley (SD) rats chosen were male and weighed approximately 160 g. The rats were caged and raised under 12-h light-dark cycle in the specific pathogen free (SPF)-grade animal room of the experimental animal center of PLA General Hospital. Standard rat chow and water were provided for the rats. Our study was carried out according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health strictly. The Local Ethics Committee of the Chinese PLA Medical Academy approved the protocol (Permit Number: 2012-32).

To establish the animal model of hepatic fibrosis, 48 male SD rats were randomly divided into control ($n = 12$) and dimethylnitrosamine (DMN) -treatment groups (dimethyl nitrosamine, Tianjin Chemical Reagent Research Institute, Tianjin, China) ($n = 36$). In the DMN-treatment group, all of the rats received intraperitoneal injection with DMN and saline mixture at a dose of 10 mL/kg for three consecutive days per week for three weeks. The DMN group was further subdivided into three groups ($n = 12$): Model group, DMN only; Transplantation group, 5×10^6 UC-MSCs administered in 0.1 mL of normal saline by tail vein injection seven days after the first DMN treatment; Control group, DMN treatment + PBS administration

by tail vein. After 3 weeks, the rats were euthanized and serum samples collected for biochemical tests. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were detected using kits from Sigma. The right lobes of the livers were excised and soaked in 10% neutral formaldehyde for histology.

Evaluation of liver inflammation and fibrosis

Hematoxylin and eosin (HE) and Masson's trichrome staining were performed to assess semi-quantitatively the pathogenesis of liver inflammation and fibrosis. An expert pathologist was arranged to assess the condition of liver inflammatory cell infiltration. This was a single-blind test and was based on the microscopic characteristics of the nucleus. The liver inflammation assessment focused on the number of polymorphonuclear leukocytes per 100 hepatocytes. Liver fibrosis was also assessed by an expert pathologist who graded it into five stages: Stage 0, normal connective tissue (no fibrosis); Stage 1, fibrous portal expansion; Stage 2, periportal fibrosis with short septa extending into lobules or rare porto-portal septa (intact architecture); Stage 3, fibrous septa reaching adjacent portal tracts and the terminal hepatic venule (architecture distortion, but no obvious cirrhosis); Stage 4, diffuse nodular formation (cirrhosis).

Micrographs were acquired using a Nikon Microphot-FXA microscope equipped with a Nikon Digital Camera DXM1200F. Digital images of HE and Masson staining were analyzed using the Image-Pro Plus software.

Immunohistochemical examinations

Paraffin-embedded liver sections were deparaffinized, using xylene and alcohol. After hydration, slides were treated with a primary polyclonal antibody raised against α -SMA (1:200, Abcam, Cambridge, MA, United States), followed by biotinylated secondary antibody. Detection was carried out with streptavidin peroxidase, and the integrated optical intensity of α -SMA was semi-quantified using the Image-Pro Plus software.

Enzyme-linked immunosorbent assays

IL-4 and IL-10 levels in serum and cell culture supernatants were measured using ELISA kits (R&D Systems), according to the manufacturer's instructions. Samples were assessed in duplicate, and the absorbance was read at 450 nm on a Thermo Fisher microplate reader (Massachusetts, America). Cytokine concentrations were calculated using standard curves generated by the plate-reader's software.

Isolation, purification, culture and identification of liver macrophages

Liver macrophages were isolated from DMN-treated rats through the collagenase perfusion method. Livers were minced and immersed in Hanks balanced

salt solution at 37 °C for 30 min. Hanks solution consisted of 24 μ g/mL Liberase (Roche Diagnostics, GmbH, Penzberg, Germany) and 1.6 U/mL DNase I (Roche Diagnostics). After that, the liver pieces were homogenized and strained through a 40 μ m filter. The filtrate was then plated in RPMI containing 2% fetal calf serum on regular non-tissue medium-treated Petri dishes overnight. Non-adherent cells were washed off, and the adherent cells used for quantitation after staining with F480 monoclonal (eBioscience, San Diego, CA, United States), CD11b monoclonal (Millipore, Billerica, MA, United States) and CD206 polyclonal (eBioscience) antibodies, respectively. The purity of the macrophages was estimated by flow cytometry using F480 monoclonal antibody, and purity of > 80% was obtained. A phagocytosis test with FITC-labeled ovalbumin was used to identify liver macrophages. M2 macrophages were identified as F480+/CD206+.

To observe the effect of IL-4 on macrophage mobilization, an overdose (final concentration 250 μ g/mL) of an IL-4 monoclonal antibody (Santa Cruz) was incubated with cultured macrophages to block IL-4.

Co-culture of UC-MSCs and macrophages

Before carrying out co-culture of UC-MSCs and macrophages, macrophages isolated from liver tissues were activated with lipopolysaccharide (final concentration of 1 μ g/mL) for 18 h. Then, 1×10^5 to 3×10^5 UC-MSCs were seeded in a 24-well plate as a supporting layer, and 1×10^5 to 3×10^5 macrophages were added to the plates.

Statistical analysis

All statistical analyses were performed using the SPSS Version 16 software. Differences were considered statistically significant at $P < 0.05$. Values are presented as the mean \pm SD. For semi-quantitative analysis of histological staging, non-parametric tests (Wilcoxon test) were used; other statistical analyses were performed using an unpaired Student's *t*-test.

RESULTS

MSCs were isolated and purified successfully

MSCs were long spindle-shaped cells (Figure 1A) with potent proliferation activity. MSCs derived from the umbilical cord did not express CD11b, CD19, CD34, CD45 and HLA-DR (0.61%), which was confirmed by flow cytometry. However, high numbers of cells expressing CD44 (99.99%), CD73 (99.98%), CD90 (99.99%) and CD105 (99.97%) were observed (Figure 1B). In addition, MSCs had osteogenic, adipogenic, and chondrogenic capabilities (Figure 1C). These findings suggested that these cells were MSCs.

Liver fibrosis is alleviated by UC-MSCs transplantation

DMN is a potent hepatotoxin that causes centrilobular

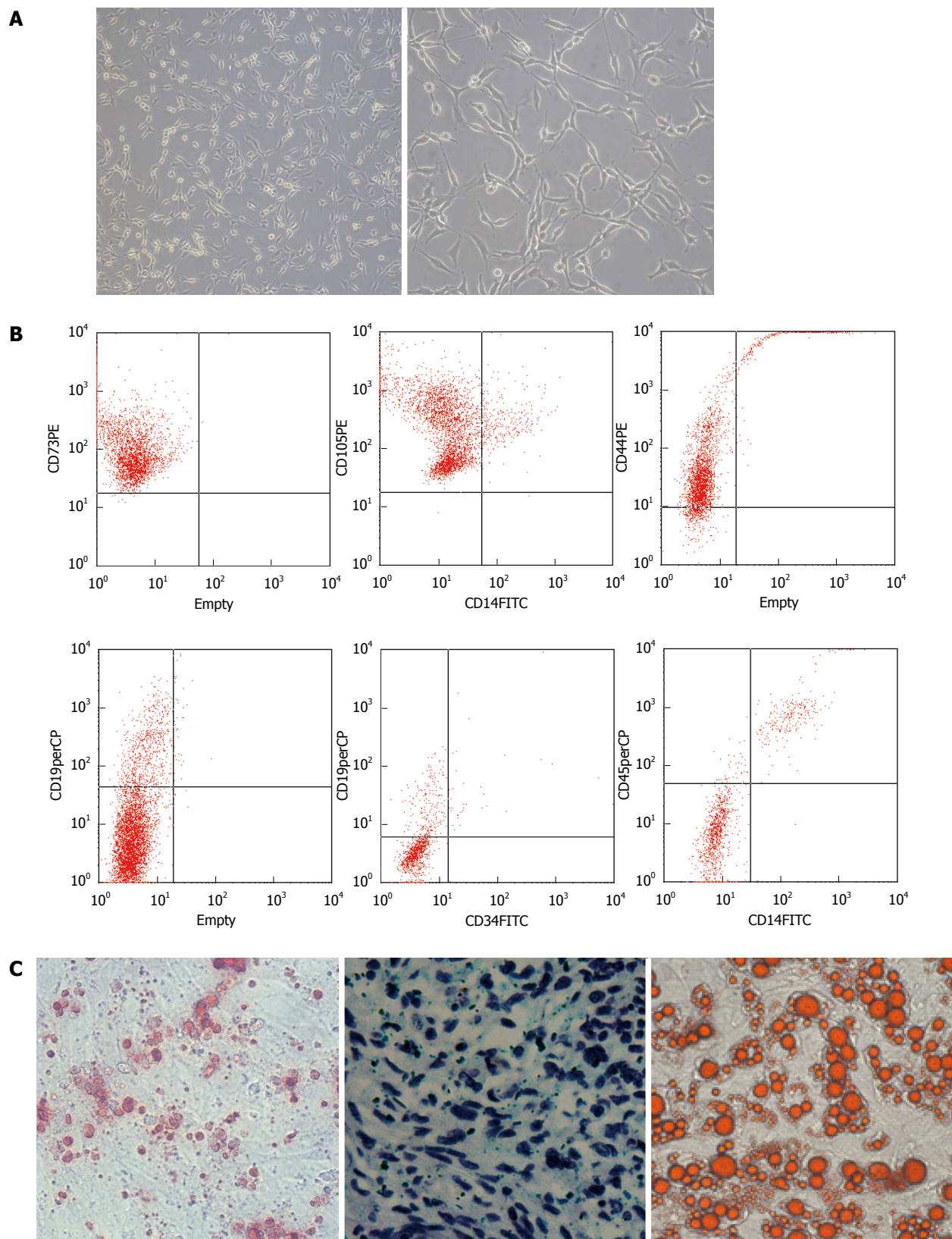


Figure 1 Mesenchymal stem cells were isolated and purified successfully. A: Mesenchymal stem cells (MSCs) are long spindle-shaped cells (right, magnification $\times 40$); B: Flow cytometry showing that umbilical cord derived MSCs express high levels of CD73, CD105 and CD44, but almost no CD19, CD34, CD45 and CD14; C: The purified MSCs were capable of osteogenesis (left, magnification $\times 40$, by Alizarin Red), chondrogenesis (middle, magnification $\times 40$, by Toluidine Blue O) and adipogenesis (right, magnification $\times 40$, by Oil Red-O).

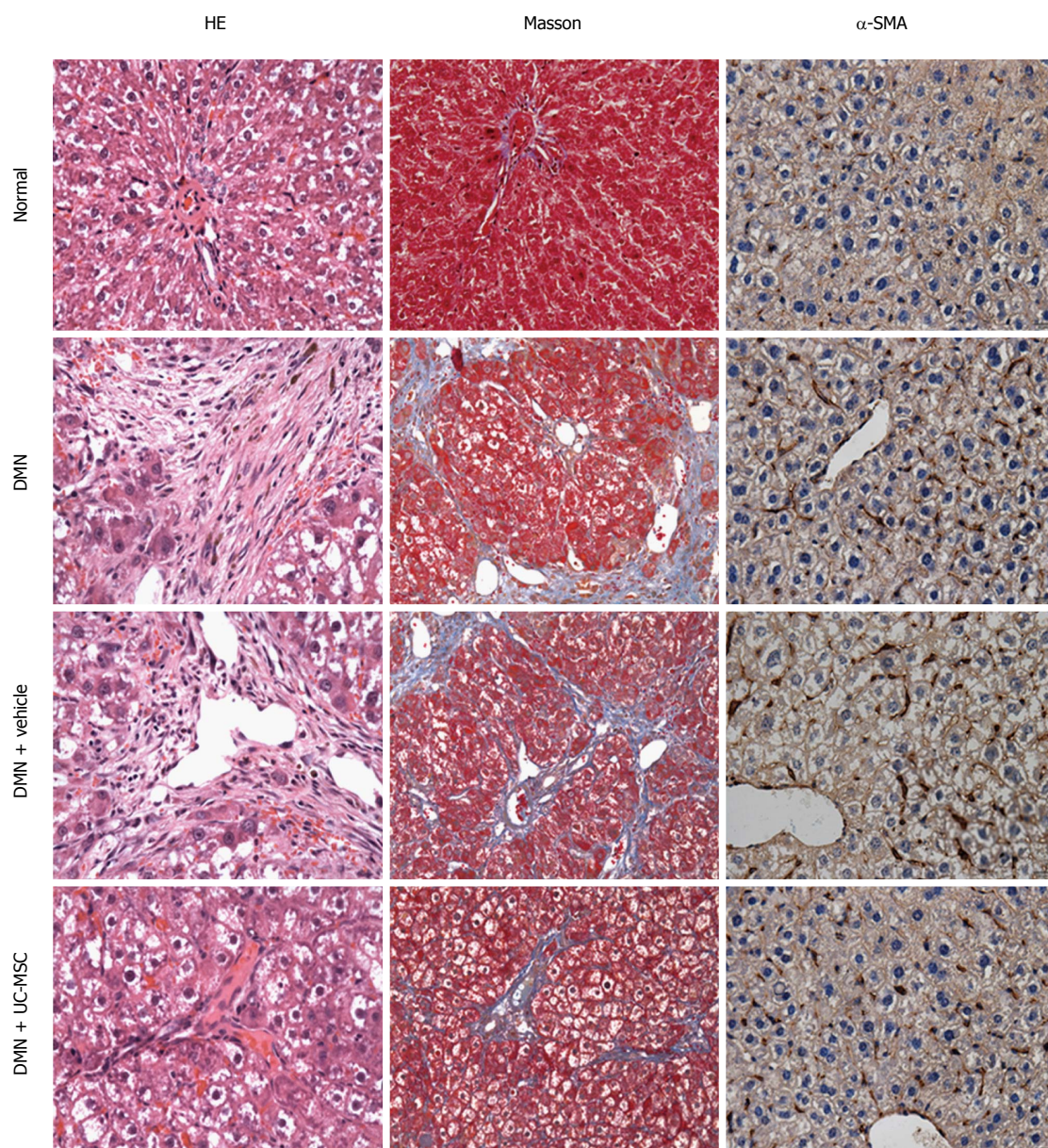


Figure 2 Umbilical cord-derived mesenchymal stem cells transplantation alleviates liver fibrosis. Hematoxylin and eosin (HE) staining of liver sections showing massive vacuolar degeneration of hepatocytes and intense neutrophil infiltration in DMN-treated rats and control rats (first column, HE staining). Masson's trichrome staining showed periportal fibrosis with fibrous septa extending into adjacent portal tracts and the terminal hepatic venule in DMN-treated rats and DMN + Vehicle animals (second column, Masson staining), suggesting the successful establishment of the animal model of hepatic fibrosis. Liver fibrosis and inflammation were significantly decreased by transfusion with UC-MSCs in UC-MSC-treated rats compared with the DMN + Vehicle group, which was characterized by short septa extending into lobules or portal-portal septa (first column, HE staining, and second column, Masson staining). Alpha-SMA is a marker of activated hepatic stellate cells, which plays key roles in the pathogenesis of liver fibrosis. Increased expression of α -SMA was observed in the model and DMN + Vehicle groups, and was decreased by UC-MSCs transfusion (third column, α -SMA immunohistochemical staining).

necrosis and nephrotoxic damage following peritoneal injection in rats. After treatment, rats were necropsied, and the right lobe of the liver was extracted from each animal for histology. When the liver tissue was processed with Masson's trichrome staining, periportal fibrosis was observed. The fibrous septa extended into the adjacent portal tracts and the terminal hepatic venule. In these DMN-treated rats, neutrophil infiltration and massive vacuolar degeneration were observed, which indicated the successful establishment of an animal model of hepatic fibrosis (Figure 2).

Liver fibrosis is initiated by inflammation, reflected by hepatocyte injury. Serum transaminase (ALT and AST) activities are known markers of such injury. In the DMN-treated rat model, ALT and AST levels were significantly increased compared with control rats ($P < 0.001$). Interestingly, these enzymes were significantly reduced after UC-MSCs transfusion, compared with rats in the DMN + Vehicle group ($P = 0.008$). Furthermore, liver fibrosis and cholestasis were significantly reduced by transfusion of UC-MSCs in the DMN-treated rats as compared with the DMN + Vehicle

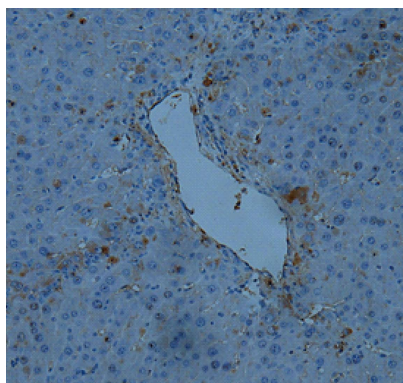


Figure 3 Umbilical cord-derived mesenchymal stem cells transfused into rats are recruited into the injured liver. Umbilical cord-derived mesenchymal stem cells (UC-MSCs) transfused into fibrotic liver animals were labeled with green fluorescent protein (GFP). Immunohistochemical detection with anti-GFP was performed to assess the distribution of UC-MSCs in the liver after DMN treatment. UC-MSCs were located in the injured liver together with infiltrated inflammatory cells (magnification $\times 40$).

group, which was characterized with shorter septa extending into lobules or portal-portal septa (Figure 2). Densitometry measurements showed that the amount of collagen increased by more than 80-fold in DMN rats compared with normal rats ($P < 0.001$); after UC-MSC transfusion, this increase was reduced by about 45% compared with control rats in the DMN model ($P = 0.021$). These results indicated that UC-MSCs could ameliorate the hepatic fibrosis induced by DMN.

UC-MSCs transfused into rats are recruited into the injured liver

It has been reported that UC-MSCs can differentiate into hepatocytes, the main cell type in the liver. We hypothesized that UC-MSCs transfused into rats could be recruited by the injured liver, differentiate into hepatocytes, and promote recovery from liver injury. To test this hypothesis, UC-MSCs were labeled with green fluorescent protein (GFP), and immunohistochemical detection was performed to assess the distribution of UC-MSCs in the liver of DMN-treated animals. As shown in Figure 3, UC-MSCs were located in the injured liver together with infiltrated inflammatory cells, indicating that UC-MSCs transfused into rats can be recruited into the injured liver.

UC-MSCs transfusion promotes KC mobilization in fibrotic liver

KCs are resident hepatic macrophages that play important roles in liver physiology by secreting inflammatory factors in response to stimulation or toxic compounds. Classical (M1) and alternative (M2) macrophages are the extreme states of macrophage phenotypes. Although M1 macrophages will trigger inflammation, such inflammation can be counterbalanced by M2 macrophages. The alternatively polarized M2 macrophages facilitate the resolution of inflammation and tissue repair.

To observe the effects of UC-MSCs on KC mobilization, UC-MSCs were transfused on day 7 into rats with liver fibrosis induced by DMN. At days 7, 14 and 21, three rats were sacrificed, respectively, and KCs were isolated, identified by positive phagocytosis test, and by CD11b and F480 expression as assessed by flow cytometry (Figure 4A-C). Furthermore, after transfusion with UC-MSCs, CD206⁺ KCs (M2) increased significantly in a time-dependent manner; the largest number of M2 cells appeared at day 21 (Figure 4D). Given that M2 cells are CD206⁺ and M1 cells are CD206⁻, these data further confirmed that UC-MSCs transfusion could promote M1 to M2 transformation in liver fibrosis induced by DMN.

UC-MSCs promote KC mobilization in vitro

We confirmed that UC-MSC transfusion promotes the mobilization of KC *in vivo*. To assess the effect of UC-MSCs on KC mobilization *in vitro*, we isolated KCs from rats treated with DMN for three consecutive weeks, and co-cultured them with UC-MSCs. KCs isolated from the same rats and cultured alone were used as the control group. Less than 4% of CD206⁺ M2 cells appeared in the KCs cultured alone; meanwhile, more than 80% M2 cells appeared in KCs co-cultured with UC-MSCs (Figure 5A-D). Given the anti-fibrogenic effect of M2 cells and the pro-fibrogenic effect of M1 cells, our data showed that UC-MSCs promoted KC mobilization not only *in vivo*, but also *in vitro*.

UC-MSCs transfusion promotes IL-4 and IL-10 production in vivo and in vitro

After transfusion of UC-MSCs into DMN rats, serum IL-4 and IL-10 levels were assessed by ELISA. Interestingly, IL-4 and IL-10 levels declined significantly after DMN treatment, and this effect was reversed by UC-MSCs transfusion. Serum IL-4 and IL-10 levels were significantly elevated in DMN-treated rats transfused with UC-MSCs compared with animals administered with DMN alone (Figure 6A and B).

In vitro, we co-cultured UC-MSCs with KCs isolated from DMN rats, and cell culture supernatants were collected for ELISA: IL-4 and IL-10 levels in KCs plus UC-MSCs were, respectively, more than 3- and 4-fold higher than the values obtained for KCs cultured alone (Figure 6C).

The consistent results between *in vivo* and *in vitro* data mean that UC-MSCs promote IL-4 and IL-10 production in KCs, which subsequently resulted in KC mobilization.

UC-MSCs secrete IL-4 and promote KC mobilization in vitro

We detected IL-4 production in cell culture medium of UC-MSCs by ELISA (data not shown), and confirmed that UC-MSCs promote KC mobilization, accompanied with elevated IL-4 production; therefore, we hypothesized that UC-MSCs secrete IL-4, which

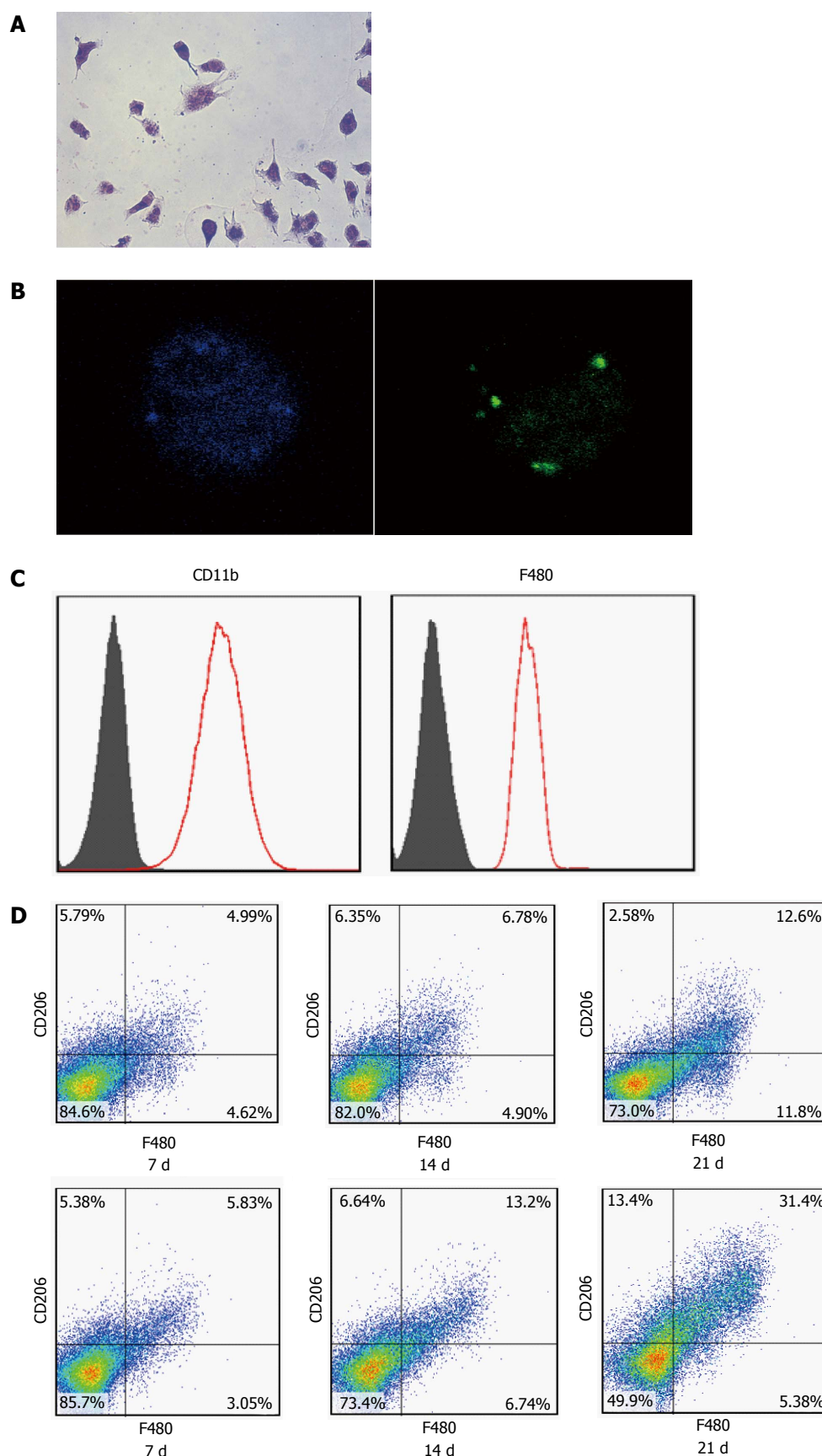


Figure 4 Umbilical cord-derived mesenchymal stem cells transfusion promotes polarization of Kupffer cells in the fibrotic liver. Kupffer cells (KCs) were isolated from the liver. The morphology of cells cultured with FITC-labeled ovalbumin showed green fluorescent (A, B) cells that were CD11b- and F480-positive, as assessed by flow cytometry analysis (C). After transfusion with UC-MSCs, CD206-positive KCs increased significantly in a time-dependent manner (D).

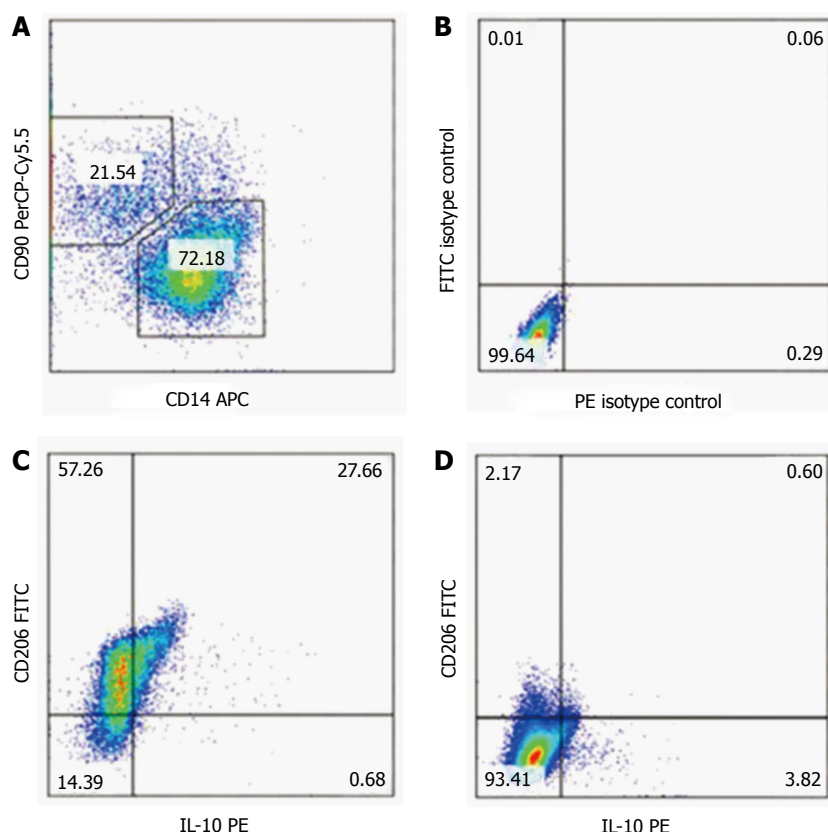


Figure 5 Umbilical cord-derived mesenchymal stem cells promote polarization of KCs *in vitro*. We isolated Kupffer cells (KCs) from rats treated with DMN for three consecutive weeks, and co-cultured them with umbilical cord-derived mesenchymal stem cells (UC-MSCs). KCs isolated from the same rats and cultured alone were set as the control group. Less than 4% of CD206 positive M2 cells appeared in KCs cultured alone; whereas, more than 80% M2 cells appeared in KCs co-cultured with UC-MSCs (A). Given the anti-fibrogenic effect of M2 cells, and the M1 cells' pro-fibrogenic effect, our data showed that UC-MSCs promoted KC polarization *in vitro*.

participates in KC mobilization. To test this, KCs were isolated from DMN-treated rats, and flow cytometry analysis showed that about 6% KC were CD206+ M2 cells; when UC-MSCs were added into the culture medium, the proportion of M2 cells increased to more than 70%. Interestingly, anti-IL-4 antibody treatment blocked the UC-MSCs effect and less than 30% of KCs transformed into M2 (Figure 7).

DISCUSSION

As a heterogeneous population of cells, MSCs have the potential for multilineage differentiation. They can be isolated from various tissues, such as blood, muscle, adipose tissue, trabecular bone and even skin. Bone marrow mesenchymal stem cells (BMSCs) can differentiate into a variety of liver cells, under appropriate culture conditions^[17-19]. Recent studies have demonstrated that BMSCs are useful to treat liver fibrosis and do not suffer from allograft rejection. BMSCs infusion is beneficial not only to ameliorate liver fibrosis, but also to reverse fulminant hepatic failure, which has been confirmed in rat models^[20]. Many clinical studies have indicated that BMSCs are safe and effective in clinical studies. They can alleviate end-stage liver disease, and improve symptoms and liver function^[21-23]. However, the invasive nature of bone

marrow aspiration might limit their clinical application. Paradoxically, some studies indicated that BMSCs have the potential to promote fibrosis^[24-26]. Thus, the application of BMSCs as a therapy for liver fibrosis remains controversial.

UC-MSCs are of particular interest because of their relatively easy accessibility and abundant source, making them a good substitute for BMSCs in future clinical studies. Indeed, UC-MSCs are reported to have greater proliferative capacity, lower immunological reactivity and lower risk of graft-vs-host disease compared with BMSCs^[5]. Rosland *et al.*^[27] reported that the rate of BMSCs spontaneous malignant transformation during culture is 45.8% (11 of 24), concluding that spontaneous malignant transformation might represent a biohazard in long-term *ex vivo* expansion of BMSCs. Similar properties of BMSCs from both human and murine origins have been reported in other studies^[28-30]. Interestingly, Tang *et al.*^[31] showed that human UC-MSCs propagating in continuous culture ultimately enter senescence and are not susceptible to spontaneous malignant transformation, suggesting the biosafety of expanding human UC-MSCs *in vitro* for use in regenerative medicine.

Recently, it has been reported that transfusion of UC-MSCs could improve significantly the symptoms of primary biliary cirrhosis, with few adverse effects^[32].

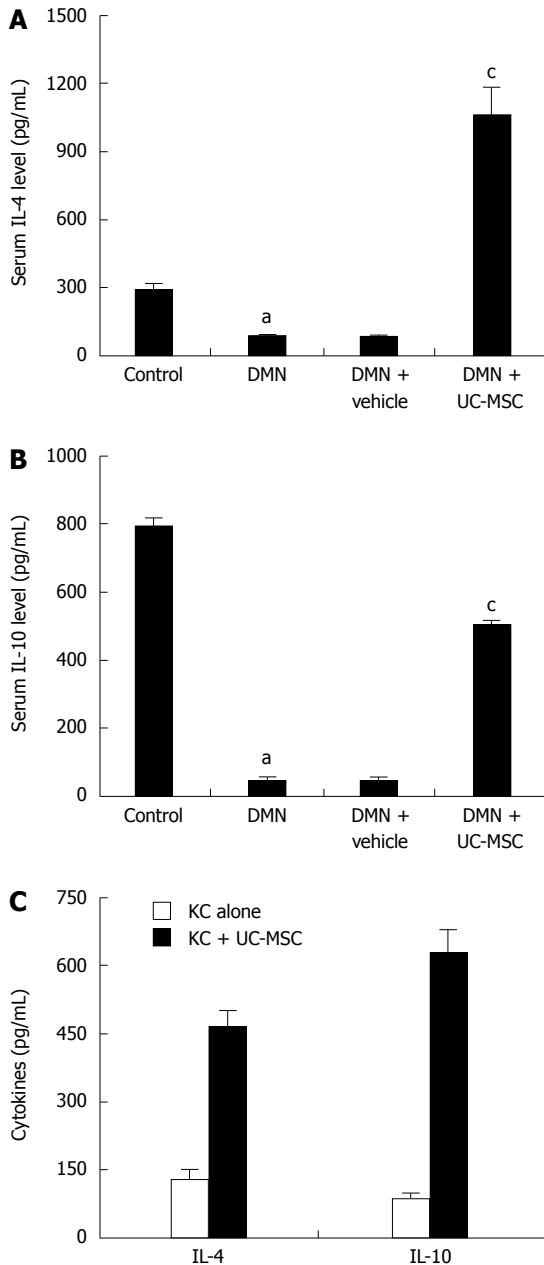


Figure 6 Umbilical cord-derived mesenchymal stem cells transfusion promotes the production of IL-4 and IL-10 *in vivo* and *in vitro*. Serum IL-4 and IL-10 levels were detected by ELISA. IL-4 and IL-10 levels declined significantly after DMN treatment, and this effect was blocked by umbilical cord-derived mesenchymal stem cells (UC-MSCs) transfusion. Serum IL-4 and IL-10 levels were elevated significantly in DMN rats transfused with UC-MSCs compared with rats treated with DMN alone (A and B, ^a $P < 0.01$ compared with the control group; ^c $P < 0.01$ compared with the DMN + vehicle group). *In vitro*, we co-cultured UC-MSCs with Kupffer cells (KCs) isolated from DMN rats, and cell culture supernatants were prepared for ELISA: IL-4 and IL-10 levels in KC plus UC-MSCs were, respectively, more than 3- and 4-fold higher than the values obtained for KC cultured alone (C). The consistent results from *in vivo* and *in vitro* experiments indicated that UC-MSCs promote IL-4 and IL-10 production in KCs, which resulted in subsequent KC polarization.

These findings suggested that UC-MSCs carry a great promise for the treatment of chronic liver disease.

MSCs have a high differentiation potential both *in vitro* and *in vivo*^[17,33]. Barry *et al.*^[33] were the first to describe the hepatic potential of MSCs, and MSC

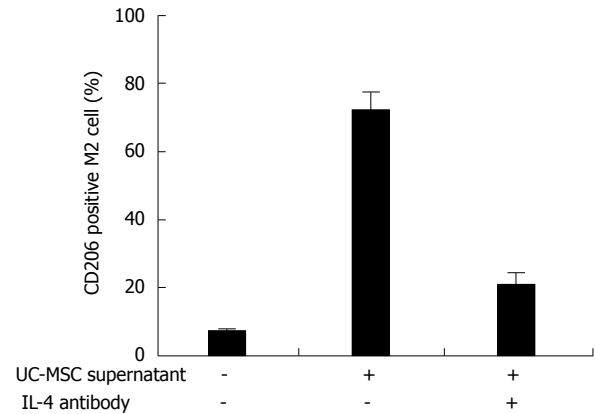


Figure 7 Umbilical cord-derived mesenchymal stem cells secrete IL-4 and promote Kupffer cells polarization *in vitro*. We isolated Kupffer cells (KCs) from DMN rats. Flow cytometry analysis showed that about 6% of the KCs were CD206 positive M2 cells; when umbilical cord-derived mesenchymal stem cells (UC-MSCs) were added into the culture medium, M2 cells increased to more than 70%. Interestingly, anti-IL-4 antibody treatment blocked the UC-MSC effect and less than 30% KCs became M2 cells.

transfusion could be a useful strategy for cellular therapy in liver fibrosis. Oh *et al.*^[34] found that two liver-specific proteins (α -feto protein and albumin) are expressed in rat bone marrow cell culture. Other studies also reported that MSCs can express albumin *in vitro*^[3,4]. In the present study, green fluorescent protein (GFP) labeled UC-MSCs were transfused into rats with liver fibrosis induced by DMN. Abundant GFP positive cells were observed in DMN-induced livers 1 wk after UC-MSCs transfusion, mostly around areas with inflammatory cell infiltration. We inferred that the UC-MSCs located in the injured liver had differentiated into hepatocytes, subsequently ameliorating the DMN-induced liver injury in rats, which further supported the concept of cell therapy for the treatment of liver injury.

The therapeutic effects of stem cell transplantation on liver fibrosis and cirrhosis have been widely investigated in mice and humans; however, the underlying mechanisms remain obscure. Depending on the cytokine composition in the tissue environment, macrophages differentiate into distinct subclasses. Classically activated macrophages (M1) differentiate in presence of Th1 cytokines (e.g., IFN- γ), or bacterial products such as lipopolysaccharide. M1 can trigger proinflammatory responses, which are needed to kill intracellular pathogens^[35]. The alternatively activated macrophages (M2) are induced by Th2 cytokines, such as IL-4 and IL-13. They are associated with Th2-type immune responses, for example, in helminth parasite infections^[35], and play an important role in protecting the organism against tissue damage^[36] during inflammation. It has been reported that mobilization of KCs from the M1 phenotype to the M2 phenotype might promote recovery from liver injury^[16]. However, we still know little about the mechanisms underlying the acquisition of the M2 phenotype.

Our data showed that UC-MSCs may promote M1 macrophage mobilization in liver fibrosis induced

by DMN not only *in vivo*, but also *in vitro*, indicating that the alleviation of liver fibrosis after UC-MSCs transfusion is partly attributed to an increase in the conversion of M1 macrophages into M2 macrophages.

A recent study by Wan *et al.*^[37] reported that polarized M2 macrophages promote M1 macrophage apoptosis *via* IL-4, uncovering a novel mechanism for M1/M2 balance regulation that relies on M2-induced M1 macrophage apoptosis.

The cytokines produced by different types of macrophages are very important for the development and function of both innate and adaptive immune responses. IL-10 is secreted by M2 macrophages^[38], and its anti-inflammatory effect has been reported in various models of acute and chronic liver injury^[39,40]. Furthermore, Suh *et al.*^[41] demonstrated that bone marrow cells can alleviate inflammation and fibrosis through the expression of IL-10. In addition, previous data^[37] identified IL-10 as the mediator of the apoptosis of M1 KCs induced by their M2 counterparts, by showing that anti-IL-10 antibodies blunt the pro-apoptotic effects of IL-4 in conditioned media. Herein, we demonstrated increased M1 macrophage mobilization and improvement of liver fibrosis following UC-MSCs transfusion; elevated IL10 production in the plasma and liver were also found. Therefore, it is plausible that UC-MSCs transfusion improves liver fibrosis *via* the following mechanism: UC-MSCs promote M1 macrophage conversion into M2 macrophages, which secrete IL-10 and subsequently increase M1 macrophage apoptosis.

IL-4 is one of the markers of M2 macrophages. Milner *et al.*^[42] found that IL-4 production leads to substantial M2 macrophage accumulation in the liver. Recent evidence has suggested an association between M2 macrophage activity and restriction of fibrosis^[36,43]. This likely explains the observation that IL-4 receptor-deficient mice cannot exhibit an intact alternative activation in KCs and will increase liver inflammation, fibrosis and death during acute schistosomiasis by *Schistosoma mansoni*^[36]. By contrast, IL-4-activated M2 macrophages improved both steatohepatitis and fibrosis during experimental and human nonalcoholic fatty liver disease^[44,45].

In this study, increased M2 macrophages in the liver treated with DMN were observed. In addition, reduced liver inflammation and liver fibrosis occurred following UC-MSCs transfusion. Finally, elevated IL-4 levels in serum and liver were also noted. These findings may help further understand how UC-MSCs mediate the repair process during liver damage, which could be associated with increased IL-4 production, accompanied by subsequent M1 macrophage mobilization into M2 counterparts.

IL-4 is also regarded as a proinflammatory cytokine, with direct cytotoxic effects on hepatocytes, as shown in a previous study by Guillot *et al.*^[46], in which lethal hepatitis was induced by transduction

with recombinant adenoviruses coding IL-4 (AdIL-4). The mortality of lethal hepatitis induced by AdIL-4 transduction was dose-dependent in that study. The observed hepatotoxicity and lack of macrophage activation with AdIL-4 transduction differ from what we observed in our study. Excessive elevation of IL-4 levels in the liver could explain the difference. Milner *et al.*^[42] considered that the hepatotoxicity of AdIL-4 might be attributed to the adenovirus vector itself.

This study is the first to assess the therapeutic value of UC-MSCs in an animal model of liver fibrosis. We found that UC-MSCs transfusion by tail vein presents satisfactory results with regard to improved liver injury and alleviated liver fibrosis. Our results suggested that the therapeutic effects of UC-MSCs on liver fibrosis rely on the activation of hepatic macrophages (KCs), which provides a partial explanation of the mechanisms of UC-MSCs-mediated therapeutic benefit in liver disease. We also found that the therapeutic effects of UC-MSCs were, at least in part, caused by their upregulation of IL-10 and IL-4 in a well-known rat model of liver fibrosis. The application of UC-MSCs might provide a powerful new tool for cell therapy of liver fibrosis.

COMMENTS

Background

Umbilical cord-derived mesenchymal stem cells (UC-MSCs) possess an excellent proliferative potential. Their low immunogenicity and ease of preparation provide these cells with a sound basis for their application in future clinical studies.

Research frontiers

Currently, stem cell therapy is considered a promising treatment for various liver diseases. Increasing evidence suggests that MSCs contribute to the direct production of new hepatocytes. Among MSCs, UC-MSCs possess an excellent proliferative potential. Previous studies have shown that UC-MSCs are a well-tolerated therapy. They have the potential to improve liver function and reduce ascites and mortality, especially in hepatitis B virus patients with decompensated liver cirrhosis and liver failure.

Innovations and breakthroughs

This study was the first to assess the therapeutic value of UC-MSCs in an animal model of liver fibrosis. UC-MSCs transfusion could improve liver injury and alleviate liver fibrosis. The therapeutic effects of UC-MSCs on liver fibrosis rely on the activation of hepatic macrophages (Kupffer cells, KCs), which probably delineate partly the mechanisms of UC-MSCs-mediated therapeutic benefit in liver diseases. The authors also found that the therapeutic effects of UC-MSCs were, at least in part, caused by their upregulation of IL-10 and IL-4, in a well-known rat model of liver fibrosis.

Applications

The application of UC-MSCs might provide a powerful new tool for cell therapy of liver fibrosis.

Terminology

Transforming growth factor β 1 (TGF- β 1) is a potent fibrogenic cytokine, playing an important role in the activation of fibrogenic myofibroblasts. In fibrosis, its major source is the KCs (liver resident macrophages). Increased levels of TGF- β 1 are responsible for scar tissue formation. Anti-smooth muscle α -actin is a marker of activated hepatic stellate cells, and HSCs play key roles in the

pathogenesis of liver fibrosis.

Peer-review

This paper adds to the existing understanding of the use of stem cells to treat liver fibrosis.

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Retrospective Cohort Study

Development and validation of a risk score for advanced colorectal adenoma recurrence after endoscopic resection

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Author contributions: Facciorusso A designed the study, performed the statistical analysis and wrote the paper; Muscatiello N and Di Maso M performed the treatment procedures and collected the data; Vendemiale G and Serviddio G revised the paper.

Institutional review board statement: This study was approved by the Institutional Review Board of the University of Foggia for retrospective evaluation of de-identified patients.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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Abstract

AIM: To develop and validate a risk score for advanced colorectal adenoma (ACA) recurrence after endoscopic polypectomy.

METHODS: Out of 3360 patients who underwent colon polypectomy at University of Foggia between 2004 and 2008, data of 843 patients with 1155 ACAs was retrospectively reviewed. Surveillance intervals were scheduled by guidelines at 3 years and primary endpoint was considered 3-year ACA recurrence. Baseline clinical parameters and the main features of ACAs were entered into a Cox regression analysis and variables with $P < 0.05$ in the univariate analysis were then tested as candidate variables into a stepwise Cox regression model (conditional backward selection). The regression coefficients of the Cox regression model were multiplied by 2 and rounded in order to obtain easy to use point numbers facilitating the calculation of the score. To avoid overoptimistic results due to model fitting and evaluation in the same dataset, we performed an internal 10-fold cross-validation by means of bootstrap sampling.

RESULTS: Median lesion size was 16 mm (12-23) while median number of adenomas was 2.5 (1-3), whereof the number of ACAs was 1.5 (1-2). At 3 years after polypectomy, recurrence was observed in 229 ACAs

(19.8%), of which 157 (13.5%) were metachronous neoplasms and 72 (6.2%) local recurrences. Multivariate analysis, after exclusion of the variable "type of resection" due to its collinearity with other predictive factors, confirmed lesion size, number of ACAs and grade of dysplasia as significantly associated to the primary outcome. The score was then built by multiplying the regression coefficients times 2 and the cut-off point 5 was selected by means of a Receiver Operating Characteristic curve analysis. In particular, 248 patients with 365 ACAs fell in the higher-risk group (score ≥ 5) where 3-year recurrence was detected in 174 ACAs (47.6%) whereas the remaining 595 patients with 690 ACAs were included in the low-risk group (score < 5) where 3-year recurrence rate was 7.9% (55/690 ACAs). Area under the curve of the model was 0.81 (0.72-0.86) with an overall classification error rate of 0.09. The model was finally validated by means of 10-fold cross validation.

CONCLUSION: Our study provides support for the use of a novel risk score as a clinical predictor of ACA recurrence after colon polypectomy.

Key words: Advanced colorectal adenoma; Colonoscopy; Colorectal cancer; Polypectomy; Survival

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Core tip: This is a retrospective study to develop and validate a novel risk score aimed at predicting advanced colorectal adenoma (ACA) recurrence after endoscopic polypectomy. The score based on lesion size, number of ACAs and grade of dysplasia, considering 5 as cut-off point, defined two different risk groups: high-risk group (score ≥ 5) with a 3-year recurrence rate of 47.6% and low-risk group (score < 5) with a 3-year recurrence rate of 7.9%. Further evidence, provided by large randomized controlled trials, is necessary in order to completely address this important issue.

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INTRODUCTION

Colorectal cancer (CRC) is the second cause of cancer-related mortality in developed countries and the third most common malignancy worldwide^[1]. However, CRC death rates have declined by approximately 3% per year during the past decade, which is most likely due to the improvement of screening programs and

standard treatments^[1].

Early interruption of adenoma-carcinoma sequence, by means of screening and surveillance programs, is widely recognized to prevent CRC occurrence and to have a significant influence on patient survival^[2,3].

According to current guidelines, surveillance colonoscopy should be repeated at 5-10 years after endoscopic resection of a single (or two) lesions < 1 cm presenting tubular features or low-grade dysplasia at histology, while follow-up should be scheduled at 3 years in cases of advanced colorectal adenomas (ACAs)^[4], defined by at least one of the following: ≥ 1 cm in diameter, villous component and high-grade dysplasia (HGD), namely those features determining an higher risk of progression to carcinoma^[5-7].

However, even in presence of ACAs, recurrence rates widely vary on the basis of several baseline clinical variables and ACA-related features such as size, number and histological characteristics^[7] in addition to type of endoscopic resection (whether en bloc or piecemeal)^[8].

Therefore, an accurate risk stratification model aimed at suggesting the appropriate post-polypectomy surveillance remains an unmet need in gastrointestinal endoscopy^[6-10].

A number of recent studies have explored the impact of different risk factors on local recurrence after ACA resection but the interaction between these factors is still unclear^[5-7,9,11].

Aim of this study is to develop and validate an easy-to-use numeric score point able to accurately predict ACA recurrence after colon polypectomy in order to guide the decision for a more correct and accurate follow-up schedule "tailored" to patient characteristics.

MATERIALS AND METHODS

Patients

Between Jan 2004 and Dec 2008 about 3360 patients underwent colonoscopic polypectomy at University of Foggia and among them data of 843 patients diagnosed with ACA was retrieved. This timespan corresponded to the period when polypectomy was performed conventionally at our center, before introducing a novel technique using polidocanol injection described in a recent paper published by our group^[12]. Institutional Review Board approbation for retrospective analysis of de-identified patients' data was obtained.

Inclusion criteria to our study were: complete adenoma resection, retrieval of resected lesion for pathological analysis, no previous diagnosis of CRC or familiar hereditary polyposis syndromes, exclusion of inflammatory bowel disease, complete follow-up data.

All colonoscopies were performed by two board-certified gastroenterologists (M.d.M., N.M.) and written informed consent was obtained from all patients before the procedure.

Resection technique

All colonoscopies were performed under deep sedation using Propofol (Diprivan[®], AstraZeneca, London, United Kingdom) monitored by a board-certified anesthesiologist with an Olympus CF-230 or CF-240 video colonoscope, following cleansing of the bowel using a polyethylene glycol-electrolyte solution (Selg-Esse[®], Promefarm, Bergamo, Italy). Bowel preparation was split between the evening before and the morning of the procedure.

The interventional endoscopic techniques adopted at our center has been described elsewhere^[10,12]. Briefly, a disposable injection needle (Innoflex[®], Innovamedica, Milan, Italy) was inserted at one edge of the lesion for submucosal injection with 9 mL of saline with 1 mL of adrenaline 1:10.000 (Adrenalina, SALF, Bergamo, Italy). The volume of solution injected was dependent on the adenoma size. After the submucosal injection, the polyp was cut with a disposable electrosurgical snare (Rotable Snare[®], Boston Scientific, Natick, MA, United States) placed over the elevated tissue and connected to the ERBE electrosurgical unit (VIO 300; ERBE, Tübingen, Germany) set to Endocut Q, Effect 3. No other ablative techniques in addition to snare resection were needed.

En bloc resection was performed whenever feasible, otherwise (in cases where the lesion was too large) piecemeal resection was undertaken. Complete resection was defined as no remaining adenomatous tissue after endoscopic mucosal resection.

ACAs were identified according to Paris classification as: polypoid pedunculated type (0-1p), sessile (0-1s), non-polypoid (0-II a, 0-II b and 0-II c)^[13,14].

All resected specimens were retrieved for histopathological analysis, and classified as tubular, tubulovillous, villous or serrated adenomas.

Patients were hospitalized for observation for 24 h, had the procedure in the day hospital or underwent ambulatorial colonoscopy, depending on the complexity of the procedure and comorbidity. In each case the monitoring protocol was the same.

Follow up

All 843 recruited patients underwent follow-up colonoscopies at our Institution. Surveillance intervals were scheduled by guidelines at 3 years in the case of en bloc resection and after 3 mo in the case of piecemeal resection, since all the patients included in the study presented advanced adenomas^[4].

Recurrence was assessed by the endoscopist during follow-up, including in this definition both local recurrence (in the same site of a previous polypectomy) and the occurrence of metachronous distant polyps^[7,10].

As described elsewhere, adverse event rates (such as bleeding or perforation) were evaluated during the procedure and, in order to capture delayed bleeding, at 24 h, 7, 10 and 14 d by means of ambulatory visits and telephone calls^[12].

Only cases of significant bleeding, those requiring

interruption of the operation to perform hemostasis or thermal treatment using coagulation with snare tip or application of clips (Resolution Clip; Boston Scientific, Natick, United States), were reported^[12].

Statistical analysis

Patients characteristics were summarized using conventional statistics, like median and interquartile range (IQR) for continuous variables and absolute frequencies and percentages for categorical data. Three-year recurrence was the main outcome measure. Baseline factors with a potential prognostic effect on recurrence were initially analyzed by means of uni/multivariate logistic regression test. The effect of continuous variables on recurrence rate was assessed for each variable by forming four groups at its quartiles. When the respective regression test was significant, a spline-based approach was applied to assess the functional form of the variable on recurrence^[15]. Based on this graphical representation a clinically sensible and applicable dichotomization of the respective variable was applied.

Variables with $P < 0.05$ in the univariate analysis were entered as candidate variables into a stepwise regression model (conditional backward selection). The regression coefficients of the Cox regression model were multiplied by 2 and rounded in order to obtain easy to use point numbers facilitating the calculation of the score. A Receiver Operating Characteristic (ROC) curve analysis was conducted aimed at identifying the more accurate cut-off points for the risk score.

The performance of the model was evaluated with the area under the curve (AUC) and error rate. To avoid overoptimistic results due to overfitting, we tested the performance of our model by means of 10-fold cross validation. Ten-fold cross-validation refers to the process of dividing the original patient sample into 10 equal groups, then removing 1 group, used as validation sample, and reconstructing the model using the reduced sample set. The new model is then tested for predictive accuracy against the excluded fraction, the process is repeated 10 times (each time with a different excluded subset). Finally, ten-fold cross-validation is repeated 250 times by means of bootstrapping to reduce the effect of random splits, and an overall c-index and error rate is calculated^[10].

The analysis was performed using R Statistical Software (Foundation for Statistical Computing, Vienna, Austria) and significance threshold was established at the 0.05 level (two-sided).

RESULTS

Patients and safety data

Baseline characteristics of the whole study population of 843 patients with 1155 ACAs who underwent colon polypectomy are reported in Table 1.

Median age was 58 (IQR 52-67) and most patients

Table 1 Baseline characteristics of patients

| Variable | All patients (n = 843) |
|---|------------------------|
| Age (yr) | 58 (52-67) |
| Gender | |
| Male | 522 (61.9) |
| Female | 321 (38.1) |
| BMI | 25 (22-28) |
| ASA score | 2 (1-3) |
| Lesion size (mm) | 16 (12-23) |
| Number of adenomas | 2.5 (1-3) |
| Number of ACAs | 1.5 (1-2) |
| Morphology ¹ pedunculated (Paris 1p) | 473 (40.9) |
| Sessile (Paris 1s) | 458 (39.6) |
| Nonpolypoid (Paris 0-II a, 0-II b, 0-II c) | 224 (19.5) |
| Location ¹ | |
| Right side of the colon | 431 (37.3) |
| Left side of the colon | 724 (62.7) |
| Type of resection ¹ | 937 (81.1) |
| <i>En bloc</i> piecemeal | 218 (18.9) |
| Histology ¹ | |
| Tubular | 436 (37.7) |
| Tubulo-villous | 632 (54.7) |
| Villous | 87 (7.6) |
| Histologic grade of dysplasia ¹ | |
| Low grade | 878 (76.1) |
| High grade | 277 (23.9) |

¹Percentages computed on the total number of ACAs (n = 1155). Continuous variables are expressed as median (interquartile range) whereas categorical ones as absolute number (percentage). BMI: Body mass index; ASA: American Society of Anaesthesiology; ACA: Advanced colorectal adenoma.

were male (61.9%) with median Body Mass Index (BMI) and American Society of Anaesthesiology (ASA) score of 25 (22-28) and 2 (1-3), respectively.

Median lesion size detected was 16 mm (12-23) while median number of adenomas was 2.5 (1-3), whereof number of ACAs was 1.5 (1-2).

Polyps were pedunculated (Paris 0-1p) in 40.9%, sessile (Paris 0-1s) in 39.6% whereas non-polypoid lesions (Paris 0-II a, 0-II b and 0-II c) accounted for 19.5% of the 1155 ACAs detected.

A little over one third of ACAs were located in the right colon (37.3%) with tubule-villous as the most frequent histology (54.7%).

Out of 1155 ACAs detected, *en bloc* resection was feasible in 937 (81.1%) cases.

Neither procedure-related deaths nor transmural burn syndromes were reported. Immediate bleeding was experienced by 51 patients (6%). All immediate bleeding events clinically presented with small amount of blood and none of the patients required hospitalization or transfusion.

Delayed bleeding rate was 19/843 (2.2%) and no clip application was needed to control delayed bleeding events. Free perforation was observed in 2 patients (0.2%), both successfully treated with surgery.

Predictors of recurrence

At 3 years after polypectomy, recurrence was observed in 229 ACAs (19.8%), of which 157 (13.5%)

Table 2 Univariate analysis of risk factors for 3-year recurrence

| Variables | Odds ratio (95%CI) | P-value |
|---|--------------------|---------|
| Age (reference ≤ 55 yr) | 1.31 (0.88-1.44) | 0.14 |
| Gender (reference female) | 1.47 (0.87-1.88) | 0.09 |
| Size (reference ≤ 15 mm) | 2.84 (1.75-4.19) | < 0.001 |
| Number of ACAs (reference 1) | 2.69 (1.88-4.53) | < 0.001 |
| Morphology (reference pedunculated) | | 0.01 |
| Sessile | 1.96 (1.21-2.43) | |
| Nonpolypoid | 2.43 (1.14-3.26) | |
| Location (reference right side colon) | 1.18 (0.76-1.35) | 0.57 |
| Type of resection (reference <i>en bloc</i>) | 8.49 (3.87-11.47) | < 0.001 |
| Histology (reference Tubular) | | 0.07 |
| Tubulo-Villous | 1.49 (0.47-5.18) | |
| Villous | 1.73 (0.68-4.45) | |
| Grade of dysplasia (reference low-grade) | 3.25 (1.23-5.60) | < 0.001 |

ACA: Advanced colorectal adenoma.

were metachronous neoplasms (150 ACAs and 7 adenocarcinomas) and 72 (6.2%) were local recurrences (70 ACAs and 2 adenocarcinomas).

Univariate logistic regression selected the number of ACAs, lesion size, morphology, type of resection, and grade of dysplasia as significant predictors of 3-year recurrence (Table 2). The same variables resulted significant predictors of both local recurrence and metachronous polyps occurrence when stratifying the regression analysis by recurrence pattern (data not shown).

Stepwise regression model

The significant parameters "number of ACAs", "lesion size", "morphology", and "grade of dysplasia" were then entered into multivariate regression analysis. The variable "type of resection" was preliminarily excluded due to its collinearity with other parameters (mainly ACA size and morphology).

After stepwise removal of the variable "ACA morphology", which did not result significant in multivariate setting ($P = 0.51$), ACA size, number and grade of dysplasia remained significant predictors of 3-year recurrence. The calculated regression coefficients were multiplied times 2 and rounded in order to facilitate the calculation of the score.

As described in Table 3, patients were given 4 points in presence of HGD, whereas lesions > 15 mm and multiple ACAs determined 3 and 2 additional score points, respectively (Table 3).

Cut-off selection for the risk score

We then calculated the risk score for all the recruited patients and performed an ROC curve analysis in order to select the more accurate cut-off point able to stratify the study population according to the recurrence score (Figure 1). ROC analysis showed a score point of 5 as the value at higher specificity and sensitivity for 3-year ACA recurrence rate (Figure 1). In particular, 248 patients with 365 ACAs fell in the high-risk group (score

Table 3 Results of multivariate stepwise backward regression analysis of prognostic factors for 3-year recurrence

| Variable | Odds ratio (95%CI) | Regression coefficient | Score points ¹ | P-value |
|--------------------|--------------------|------------------------|---------------------------|---------|
| Grade of dysplasia | | | | |
| Low-grade | 1 | | - | |
| High-grade | 4.25 (2.11-7.5) | 1.93 | 4 | < 0.001 |
| Size | | | | |
| ≤ 15 mm | 1 | | - | |
| > 15 mm | 3.96 (1.87-7.55) | 1.61 | 3 | < 0.001 |
| Number of ACAs | | | | |
| 1 | 1 | | - | |
| > 1 | 3.22 (2.19-5.39) | 1.21 | 2 | < 0.001 |

¹The regression coefficients were multiplied by 2 and rounded in order to facilitate the calculation of the score. ACA: Advanced colorectal adenoma.

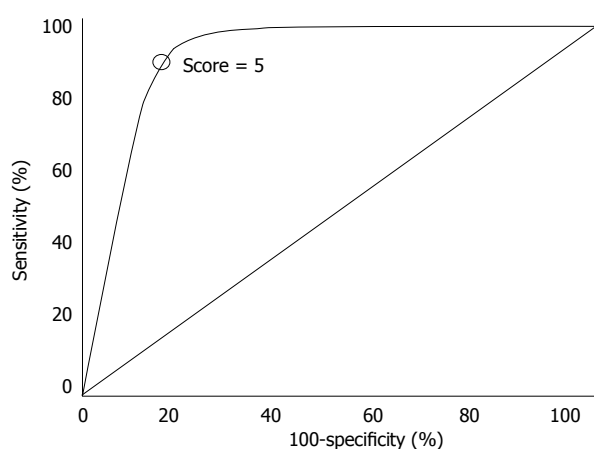


Figure 1 Receiver operating characteristic curve analysis aimed at identifying the cut-off point for the score. Receiver operating characteristic curve analysis identified the value 5 as the more sensitive and specific cut-off point for the recurrence score.

≥ 5) where 3-year recurrence was detected in 174 ACAs (47.6%) whereas the remaining 595 patients with 690 ACAs were included in the low-risk group (score < 5) where 3-year recurrence rate was 7.9% (55/690 ACAs). AUC of the model was 0.81 (0.72-0.86) with a classification error rate of 0.09.

Model validation

The model was tested by means of ten-fold cross validation. The original patient sample was partitioned into 10 equal groups, then 1 group was randomly removed each time and used as validation sample while reconstructing the model using the remaining sample set. Cross-validation thus consisted in testing each of these reduced sample sets for predictive accuracy against the excluded fractions. Finally, ten-fold cross-validation was repeated 250 times by means of bootstrapping to reduce the effect of random splits, and an overall AUC and error rate were calculated. This validation method resulted in an AUC of 0.79 (95%CI: 0.72-0.83) and in an overall error rate of 0.12 for our model (Figure 2).

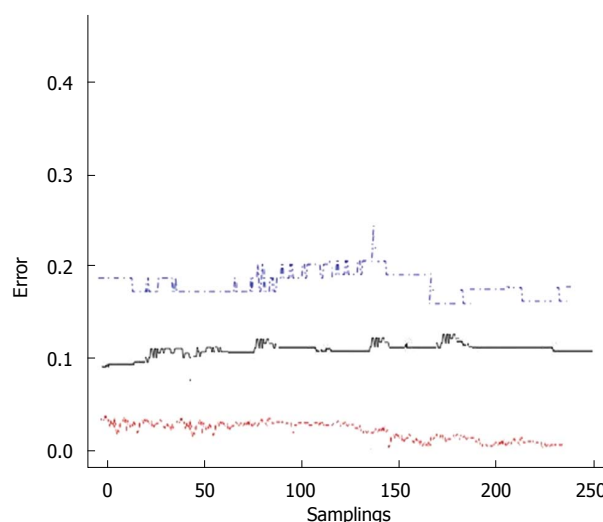


Figure 2 Ten-fold cross validation error rate. The overall average error rate was 0.12. Red line indicates error rate for recurrence prediction, blue line indicates error rate for non-recurrence prediction, black line the average error rate.

DISCUSSION

Colorectal cancer represents a major health problem and screening colonoscopy with removal of detected adenomas has proven an effective strategy to decrease CRC incidence and mortality^[3,4].

Since only 6% of patients with adenoma will develop CRC^[4], a number of recent studies have focused on the definition of risk factors for recurrence in order to identify the proper time interval from index colonoscopy to the next examination^[5,6,11].

A group of experts has recently updated the guidelines of colonoscopy surveillance after colon polypectomy^[4], but tailoring the frequency and time intervals of follow-up remains an unmet need.

Advanced colorectal adenomas (those ≥ 1 cm and/or with villous component and/or HGD) are well-known to present higher risk of adenoma recurrence after polypectomy and to more frequently develop into adenocarcinoma^[5,16,17]. However, ACAs represent a wide variety of lesions with very different recurrence rates after colon polypectomy or likelihood to degenerate^[6,11].

In a recent paper, Seo *et al*^[6] demonstrated in a large retrospective series of ACAs that the presence of 2 or more unfavorable features and piecemeal resection determine an higher risk of recurrence. Unfortunately, an accurate individualization of the risk was not possible since the exact weight of each feature and the interaction among them could not be captured by the conventional logistic regression applied by the authors. On the other hand, Martínez *et al*^[7] showed in a pooled-data analysis that 7.4% of patients identified as at low risk by current guidelines finally developed ACA or invasive cancer, thus clearly claiming among the conclusions of their paper the need for a formal prediction model able to determine the combination of

factors which would maximally distinguish the risk of recurrence.

Aim of this study was therefore to establish an objective and simple tool to define the recurrence risk for ACA patients and consequently to guide the decision process for the surveillance protocol.

We found that baseline ACA size > 15 mm, presence of multiple ACAs and high-grade dysplasia were associated to higher risk of adenoma recurrence at 3 years (Table 3).

These findings are in keeping with the published literature^[6,11,18,19]. It should be noted that histology was not selected by the multivariate model, probably because tubular ACAs are likely to present in greater sizes since, by definition, adenomas > 10 mm are to be considered “advanced” regardless of histology, whereas villous adenomas are always considered advanced, regardless of size. Lesion size hence probably “masked” the impact of histological pattern on final outcomes.

On the basis of these results, we developed a risk score by using the regression coefficients of these variables in our multivariate regression model. Once selected the value 5 as an accurate cut-off value for the point score, our model identified two groups at different risk of ACA recurrence.

Taking a closer look at our data, the low-risk group (when the total score was < 5) showed a 3-year ACA recurrence rate of 7.9% (55/690 ACAs) whereas the higher-risk group (score ≥ 5) experienced recurrence at 3 years in 47.6% of detected lesions (174/365).

We think that modelling an objective risk score enabled us to overcome the final findings of previous reports^[6,20,21], which concluded that the number of predictive ACA characteristics was more important than the type of characteristic in defining recurrence risk. On the other hand, our analysis identified two different risk classes based on an objective numeric score able to take into account either the number of ACA characteristics and the type of their features.

In our study, the incidence of adenocarcinoma after polypectomy was low (0.7%), consistently with previous reports^[6,11], thus confirming the efficacy of current surveillance programs.

The findings of the current paper are of key clinical relevance for several reasons. First, our score is simple and easily applicable in a real-life clinical setting even in countries with limited healthcare resources. Second, the application of the score may be useful in better define the surveillance schedule and protect patients with low-risk features from an excessively strict follow-up. On the other hand, “tailoring” the surveillance schedule to single patient and even ACA characteristics may significantly decrease the recurrence rate in higher risk patients, actually not adequately followed-up with the current protocols.

Nevertheless, there are some weaknesses to our study. First, the retrospective nature of the report may have introduced some outcome biases as, for instance,

patients who underwent piecemeal resection were evaluated at 3 mo after polypectomy unlike those treated with en bloc resection. However, we performed the analysis considering as the sole dependent variable 3-year recurrence rate and did not consider time-to-recurrence, which could have been affected by the different follow-up schedule. Second, as all the patients were followed-up according to current guidelines, it was not possible to assess recurrence rates at different time points, unlike other studies conducted in countries where the low medical cost of colonoscopy allowed more frequent examinations^[6]. As a consequence, we may postulate that high-risk lesions could benefit from a more intensive follow-up schedule (*i.e.*, before 3 years after colonoscopy) but definitive data in such regard is lacking. Third, the single-center experience reported in the study did not allow the external validation of the model in a different cohort. Nevertheless, an internal validation by means of 250 bootstrap samplings randomly drawn with replacement from the original population, was performed. This way, both the model building process and its performance were simultaneously validated in a broad range of random samples, thus obviating the lack of an external cohort, as recently confirmed by simulation studies^[22].

In conclusion, in the current paper we propose an objective tool aimed at classifying advanced colorectal adenomas in two groups at different risk of recurrence, based on the number of ACAs, their size and the presence of high-grade dysplasia. A score point ≥ 5 (given by the combination of at least two of the aforementioned ACA features) determine a significantly higher recurrence risk at 3 years and probably calls for a stricter follow-up schedule. Further evidence, provided by large randomized controlled trials assessing recurrence rate at several time points, is necessary in order to completely address this important issue.

COMMENTS

Background

Early interruption of adenoma-carcinoma sequence, by means of screening and surveillance programs, is widely recognized to prevent colorectal cancer occurrence and to have a significant influence on patient survival. According to current guidelines, surveillance colonoscopy should be repeated at 5-10 years after endoscopic resection of a single (or two) lesions < 1 cm presenting tubular features or low-grade dysplasia at histology, while follow-up should be schedule at 3 years in cases of advanced colorectal adenomas (ACAs), defined by at least one of the following: ≥ 1 cm in diameter, villous component and high-grade dysplasia, namely those features determining an higher risk of progression to carcinoma. However, even in presence of ACAs, recurrence rates widely vary on the basis of several variables. Therefore, an objective and easy-to-use tool aimed at suggesting the appropriate post-polypectomy surveillance remains an unmet need in gastrointestinal endoscopy. In fact, a number of recent studies have explored the impact of different risk factors on local recurrence after ACA resection but the interaction between these factors is still unclear.

Research frontiers

The authors propose an objective tool aimed at classifying advanced colorectal

adenomas in two groups at different risk of recurrence, based on the number of ACAs, their size and the presence of high-grade dysplasia. Further evidence, provided by large randomized controlled trials assessing recurrence rate at several time points, is necessary in order to completely address this important issue.

Innovations and breakthroughs

The authors think that building an objective risk score allows to overcome the final findings of previous reports, which concluded that the number of predictive ACA characteristics was more important than the type of characteristic in defining recurrence risk. On the other hand, our analysis identified two different risk classes based on an objective numeric score able to take into account either the number of ACA characteristics and the type of their features. In this study, the incidence of adenocarcinoma after polypectomy was low (0.7%), consistently with previous reports, thus confirming the efficacy of current surveillance programs. The findings of the current paper are of key clinical relevance for several reasons. First, our score is simple and easily applicable in a real-life clinical setting. Second, the application of the score may be useful in better define the surveillance schedule and protect patients with low-risk features from an excessively strict follow-up. On the other hand, "tailoring" the surveillance schedule to single patient and even ACA characteristics may significantly decrease the recurrence rate in higher risk patients, actually not adequately followed-up with the current protocols.

Applications

This study provides support for the use of a novel risk score as predictor of 3-year ACA recurrence. A score point ≥ 5 implies an higher risk of recurrence.

Terminology

ACA: Advanced colorectal adenomas, namely those ≥ 1 cm and/or villous component and/or with HGD, which are well-known to present higher risk of adenoma recurrence after polypectomy and to development into adenocarcinoma. Colon polypectomy: endoscopic removal of a mucosal lesion, aimed at interrupting the adenoma-carcinoma sequence.

Peer-review

This manuscript reported a development of a novel risk score tool for colorectal adenoma recurrence after endoscopic mucosal resection and investigated the validation with relatively large sample size. The scoring tool has a good performance for predicting the recurrence and is easily applicable at the bedside. This study design involves several limitations such as retrospective database-based study and regarding follow-up time, but the authors well discussed on these matters in the manuscript.

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Retrospective Cohort Study

Visualizing the hepatic vascular architecture using superb microvascular imaging in patients with hepatitis C virus: A novel technique

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Abstract

AIM: To identify the hepatic vascular architecture of patients with hepatitis C virus (HCV) using superb microvascular imaging (SMI) and investigate the use of SMI in the evaluation of liver fibrosis.

METHODS: SMI was performed in 100 HCV patients. SMI images were classified into five types according to the vascular pattern, and these patterns were compared with the fibrosis stage. Moreover, the images were analyzed to examine vascularity by integrating the number of SMI signals in the region of interest ROI [number of vascular trees (VT)]. The number of VT, fibrosis stage, serum parameters of liver function, and CD34 expression were investigated.

RESULTS: There was a significant difference between SMI distribution pattern and fibrosis stage ($P < 0.001$). The mean VT values in each of the fibrosis stages were as follows: 26.69 ± 7.08 in F0, 27.72 ± 9.32 in F1, 36.74 ± 9.23 in F2, 37.36 ± 5.32 in F3, and 58.14 ± 14.08 in F4. The VT showed excellent diagnostic ability for F4 [area under the receiver operator characteristic

(AUROC): 0.911]. The VT was significantly correlated with the CD34 labeling index ($r = 0.617$, $P < 0.0001$).

CONCLUSION: SMI permitted the detailed delineation of the vascular architecture in chronic liver disease. SMI appears to be a reliable tool for noninvasively detecting significant fibrosis or cirrhosis in HCV patients.

Key words: Superb microvascular imaging; Number of vascular trees; Chronic liver disease; Ultrasound; Liver fibrosis; CD34

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Core tip: Superb microvascular imaging (SMI) is an innovative Doppler ultrasound technology that employs a unique algorithm to allow for the visualization of minute vessels with slow blood flow. In the present study, we identified the hepatic vascular architecture of patients with hepatitis C virus (HCV) using SMI and investigated the use of SMI in the evaluation of liver fibrosis. SMI allowed for the detailed delineation of the vascular architecture in chronic liver disease patients. Significant differences were found in the SMI pattern distribution and the fibrosis stage. SMI appears to be a reliable tool for noninvasively detecting significant fibrosis or cirrhosis in patients with HCV.

Kuroda H, Abe T, Kakisaka K, Fujiwara Y, Yoshida Y, Miyasaka A, Ishida K, Ishida H, Sugai T, Takikawa Y. Visualizing the hepatic vascular architecture using superb microvascular imaging in patients with hepatitis C virus: A novel technique. *World J Gastroenterol* 2016; 22(26): 6057-6064 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i26/6057.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i26.6057>

INTRODUCTION

Hepatitis C virus (HCV) has a high propensity to persist and cause chronic hepatitis, eventually leading to cirrhosis^[1-3]. Cirrhosis results from different mechanisms of liver injury, which lead to hepatic necroinflammation and fibrogenesis. It is histologically characterized by diffuse nodular regeneration surrounded by dense fibrotic septa with subsequent parenchymal extinction and the collapse of liver structures^[4]. Together, these effects cause the pronounced distortion of the hepatic vascular architecture, which results in increased resistance to the portal blood flow and, consequently, portal hypertension and hepatic synthetic dysfunction. The distortion of the hepatic vascular architecture is, therefore, a major determinant of hepatic repair and the regenerative capability^[5,6]. Furthermore, the evaluation of the hepatic vascular architecture is useful for assessing the chronic liver disease (CLD) state, determining treatment strategies, and elucidating the mechanisms of disease progression.

Several studies using hepatic angiography have reported that vascular tortuosity, tapering, unevenness of branching and grouping of branches are associated with the progression of CLD^[7-12]; however, angiography is an invasive medical test. Recent research has, therefore, focused on the evaluation of noninvasive methods to identify valid, flexible, and accurate methods for assessing the distortion of the hepatic vascular architecture. The ultrasound Doppler technique, a noninvasive, radiation-free technique, is widely used to observe hepatic blood flow and vascular architecture. However, there are some technical limitations associated with this technique, such as visualization of the fine vessels and low velocity blood flow^[13,14]. Contrast-enhanced ultrasound (CEUS) detects low velocity blood flow in the microcirculation and is thus able to overcome some of these limitations. However, it does have a number of drawbacks: it is not readily available, it is subject to certain restrictions regarding contrast agent use, and it places an additional cost burden on the patient.

In recent years, Toshiba Medical Systems has developed a new Doppler technique called superb microvascular imaging (SMI)^[15,16]. SMI is a microvascular flow imaging mode that is designed to improve blood flow visualization, especially slow flow signals from microscopic vessels, using a new adaptive algorithm that dramatically removes clutter while maintaining very high frame rates. In the present study, we identified the hepatic vascular architecture of patients with HCV-related CLD using SMI and investigated the use of SMI in the evaluation of liver fibrosis.

MATERIALS AND METHODS

Patients

One hundred nineteen patients with HCV-related CLD who had undergone a liver biopsy at our institution between January and November 2015 were involved in this study. HCV-related CLD was diagnosed according to the results of a histological analysis and the detection of HCV antibodies in the serum using a third-generation enzyme-linked immunosorbent assay (Abbott Labs, Abbott Park, IL, United States). Patients with a history of drug and/or alcohol abuse (alcohol consumption of ≥ 40 g/d for men, $n = 3$, ≥ 20 g/d for women over past 12 mo, $n = 3$), hepatitis B surface antigen positivity ($n = 2$), severe obesity [body mass index (BMI) > 30 kg/m², $n = 4$], severe fatty liver ($n = 4$), and other CLD, such as primary biliary cirrhosis and autoimmune hepatitis ($n = 3$), were excluded from the present study. Among these patients, 19 patients were excluded from the analysis. Finally, data were obtained from a total of 100 patients. The mean (\pm SD) age of the patients was 65.8 ± 10.4 years (range: 41-85 years). The subjects included 56 men and 44 women. The patient profiles are presented in Table 1. The study was approved by the local Ethical Committee of Iwate Medical University (H26-124). The

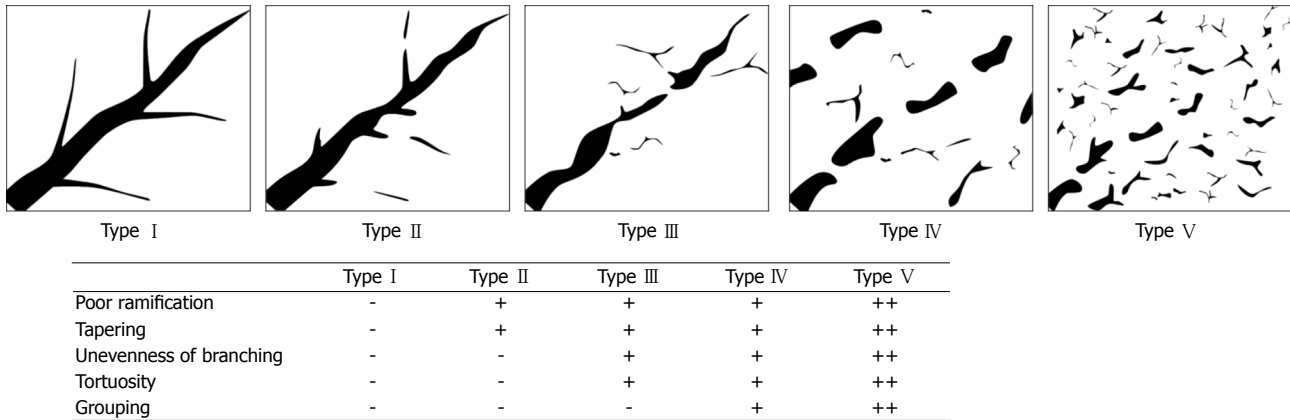


Figure 1 Superb microvascular imaging vascular patterns. Type I, clearly defined vessels with no irregularities; Type II, poor ramification and tapering of the main branches; Type III, mild tortuosity of the main branches and uneven branching; Type IV, moderate tortuosity and mild grouping of the main branches; and Type V, severe tortuosity and grouping of the main branches.

Table 1 Baseline characteristics of patients

| Variables | n = 100 |
|---|------------------|
| Sex (male/female) | 56/44 |
| Mean age (yr) | 65.8 ± 10.5 |
| BMI (kg/m ²) | 22.8 ± 3.3 |
| METAVIR score (F0/F1/F2/F3/F4) | 21/34/11/11/23 |
| T-Bil (mg/dL) | 0.6 (0.5-0.8) |
| AST (U/L) | 35.5 (25.8-53.1) |
| ALT (U/L) | 32.6 (23.6-53.8) |
| Alb (g/dL) | 3.9 (3.5-4.2) |
| PT (%) | 92.1 (82.2-98.8) |
| Plt (× 10 ⁴ /mm ³) | 14.8 (11.3-18.2) |
| HA (ng/mL) | 101 (92.1-117.5) |
| IV-c-7S (ng/mL) | 5.7 (4.4-7.8) |

The values represent the mean ± standard deviation or the median (25th-75th percentile). BMI: Body mass index; T-Bil: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; Alb: Albumin; PT: Prothrombin time; Plt: Platelet count; HA: Hyaluronic acid; IV-c-7S: Type IV collagen 7S.

patients provided their written informed consent before beginning the study in accordance with the principles of the Declaration of Helsinki (revision of Fortaleza, 2013).

Ultrasound examination technique

The ultrasound scanner Aplio500 (Toshiba Medical Systems, Otawara, Japan) combined with a 7.0 MHz linear transducer (PLT-705BT) was used. B-mode ultrasonography was performed to scan the whole liver before the SMI examination. The vascular architecture of the anterior-inferior portal vein was evaluated on monochrome SMI on the same day the patients underwent a liver biopsy. The anterior-inferior portal vein was selected because trans-abdominal ultrasound can provide a stable and high-resolution image. SMI was performed in the stable transducer position. The following settings were used for the SMI examination in all cases: the region of interest (ROI) was set to a fixed depth of 15 mm from the liver surface and the size of the ROI measured 40 mm ×

25 mm. The color velocity scale of SMI was adjusted to 1.4 to 1.6 cm/s, the color frequency was adjusted to 4 MHz, and the vascular information was enhanced by adjusting the time smooth. To avoid interobserver variability, all sonographic scanning was performed by two radiologists with > 10 years of experience in abdominal sonography and 3 mo of experience in SMI. A third radiologist with > 15 years of experience in abdominal sonography and 6 mo of experience in SMI served as a blinded expert in cases of disagreement.

According to previous hepatic angiographic reports regarding changes in vascular morphology that occur in CLD^[7-12], we classified portal vein vascular patterns into the five following types: Type I, clearly defined vessels with no irregularities; Type II, poor ramification and tapering of the main branches; Type III, mild tortuosity of the main branches and unevenness of branching; Type IV, moderate tortuosity and mild grouping of the main branches; and Type V, severe tortuosity and grouping of the main branches (Figure 1). Two additional radiologists who did not perform SMI blindly classified each of the SMI images as one of the five types according to the vascular pattern, and the frequencies of each pattern were compared with the fibrosis stages. At the end of the classification, any disagreement was discussed and resolved by a consensus.

The terminal branch of the anterior-inferior portal vein was scanned with SMI, and the maximum vessel lumen was selected for study. Flash or movement artifacts were excluded by repeated pulsed Doppler sampling of visible color signals to ensure that these signals originated from the portal veins. The images were recorded in the DICOM file format. The number of SMI vascular signals in the ROI was counted using an image analysis software program (ImageJ, National Institutes of Health, Bethesda, MD, United States)^[17]. In the present study, we referred to the number of vascular signals as vascular trees (VT). The relationships between the VT and sex, age, BMI and the results of liver function tests were examined.

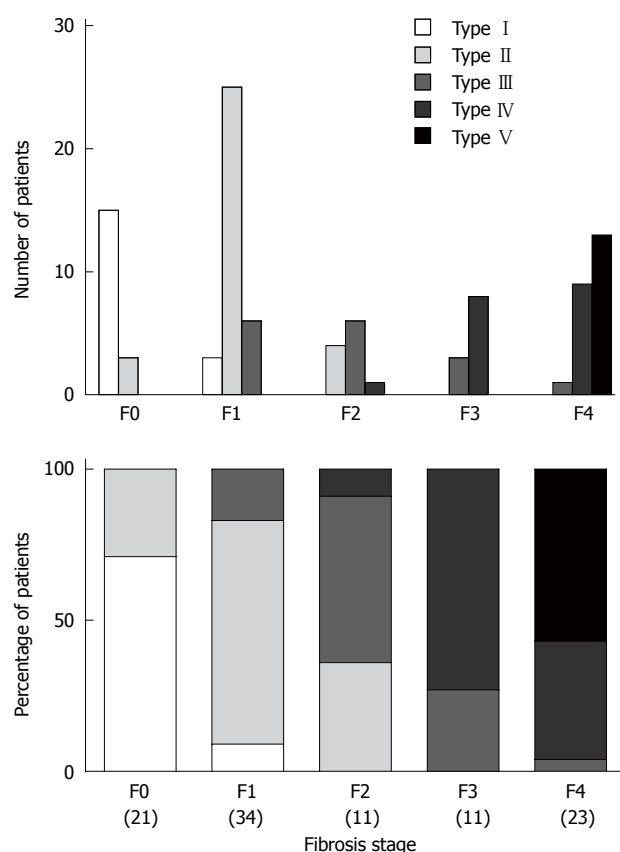


Figure 2 Distribution of the superb microvascular imaging patterns and fibrosis stages. Fisher's exact probability test demonstrated a significant difference in the SMI pattern distribution and the fibrosis stage ($P < 0.001$). SMI: Superb microvascular imaging.

Serum markers of the liver function

Biochemical tests were performed in all patients on the day of SMI using routine laboratory methods. The tests included total bilirubin (T-Bil), albumin (Alb), aspartate aminotransferase (AST), alanine aminotransferase (ALT), prothrombin time (PT), platelet count (Plt), hyaluronic acid (HA), and type IV collagen 7S (IV-c-7S).

Histology and immunohistochemistry

An echo-assisted liver biopsy was performed in the same session as SMI using a 14-G biopsy needle measuring 2.2 mm in diameter. The tissue specimens were immediately fixed in 10% buffered formalin, embedded in paraffin, and cut into 4 mm thick sections. These sections were stained with hematoxylin and eosin and Gomori trichrome stain and were assessed (according to the METAVIR score^[18]) by two highly experienced pathologists who were blinded to the results of SMI and to all of the patients' clinical, serological, and histological data. All of the liver biopsy specimens were obtained from the pathology samples at Iwate Medical University Hospital. Fibrosis was staged on a 0-4 scale: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and a small number of septa extending into the lobules; F3, numerous septa extending to the adjacent portal tracts

or terminal hepatic venules; and F4, cirrhosis.

CD34 is a 110 kDa transmembrane glycoprotein that is present on leukemic cells, endothelial cells and stem cells. CD34 is preferentially expressed on the surface of regenerating or migrating endothelial cells and is a marker of the proliferation of endothelial cells in growth during angiogenesis^[19]. Normal sinusoidal endothelial cells do not typically express CD34. However, pathological conditions can alter their phenotype and cause them to express this marker. The capillarization of the hepatic sinusoids is a well-recognized phenomenon that occurs in CLD and hepatocellular carcinoma^[20]. We performed immunohistochemical examinations to detect CD34 positivity in 61 of the patients in the present study. An anti-CD34 monoclonal mouse antibody (QEnd 10; Dako A/S, Glostrup, Denmark) was used. The avidin-biotin-peroxidase complex immunohistochemistry method (Vectastain Elite ABC Kit, Vector Laboratories Inc., Burlingame, CA, United States) was used. The number of CD34-positive capillaries and sinusoids were counted in 10 portal areas under a high-power field (200 × magnification) by two independent observers with no knowledge of the patient data. The average number was defined as the CD34 labeling index (CD34 LI). There was no significant interobserver difference; a few cases with wide differences were re-evaluated by a third observer. The present study evaluated the relationships among the fibrosis stage, the CD34 LI, and the number of VT.

Statistical analysis

Statistical tests were performed using the SPSS 12.0 software program (SPSS, Chicago, IL, United States). The values are shown as the mean ± SD, or medians (range) according to the distribution of the values. Categorical data were compared using Fisher's exact probability test. A statistical analysis of the differences in the number of VT in each fibrosis stage was performed using the Kruskal-Wallis test. The correlations between the number of VT and other parameters were assessed using Spearman's rank correlation coefficient. The clinical diagnostic ability of the VT for cirrhosis was evaluated according to the sensitivity, specificity, and area under the receiver operating characteristic (AUROC) curve. P values of < 0.05 were considered to indicate statistical significance.

RESULTS

The average time required for the SMI examination was 60.5 ± 20.1 s, and all patients cooperated with the examination. The association between the SMI pattern and fibrosis stage is presented in Figure 2. There was a significant difference in the distribution of the SMI pattern and the fibrosis stage ($P < 0.001$). The percentage of patients with advanced fibrosis (F3-4) in SMI patterns I, II, III, IV, and V was 0% (0/18), 0% (0/35), 25% (4/16), 94% (17/18), and

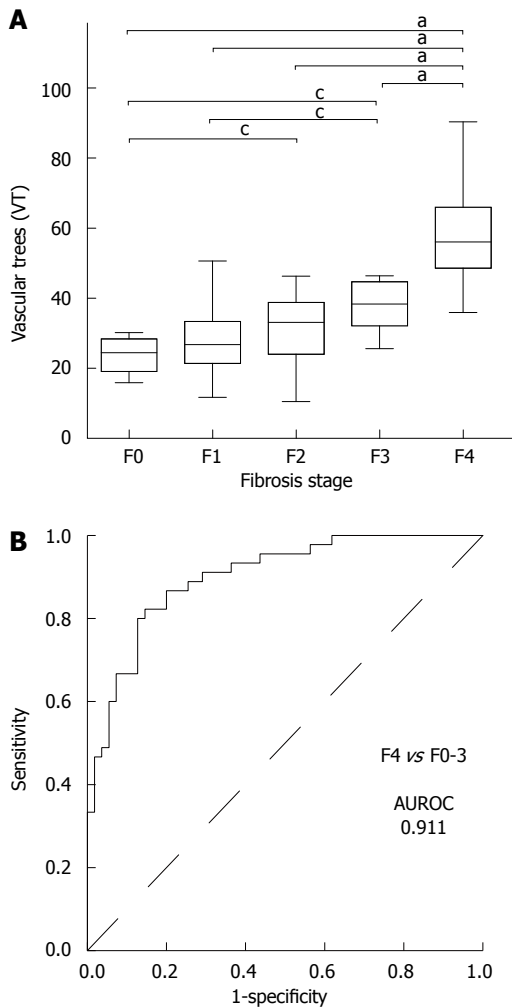


Figure 3 Vascular tree values for the different fibrosis stages in patients who underwent liver biopsy (A) and the receiver operating characteristic curve of the vascular tree for the prediction of F4 in all 100 patients. The VT values increased in proportion to the fibrosis stage ($^aP < 0.001$, $^cP < 0.01$ by Kruskal-Wallis analysis). AUROC: Area under the receiver operating characteristic; VT: Vascular trees.

100% (13/13), respectively. Conversely, mild fibrosis (F0-1) occurred in 100% (18/18), 89% (31/35), 38% (6/16), 0% (0/18), and 0% (0/13) of patients, respectively.

The mean VT values in each of the fibrosis stages were as follows: 26.69 ± 7.08 in F0 (21), 27.72 ± 9.32 in F1 (34), 36.74 ± 9.23 in F2 (11), 37.36 ± 5.32 in F3 (11), and 58.14 ± 14.08 in F4 (23). The mean VT value in F4 was significantly higher than those in F0-3 ($P < 0.001$), while the mean values in F2 and F3 were higher than those in F0 and F1 ($P < 0.01$) (Figure 3A). The ROC curve for the diagnosis of the F4 stage is shown in Figure 3B. The AUROC curve for the VT was 0.911. The most appropriate VT cut-off value for the diagnosis of F4 was 35.65, and the sensitivity and specificity were 82.2% and 85.5%, respectively.

The relationships between the VT and the clinical or laboratory parameters of the patients are shown in Table 2. The VT showed a significant negative

Table 2 Correlation between the vascular trees values and clinical or laboratory parameters

| Parameters | <i>r</i> value | <i>P</i> value |
|------------|----------------|----------------|
| Sex | 0.027 | 0.895 |
| Age | 0.213 | 0.073 |
| BMI | -0.021 | 0.836 |
| T-Bil. | 0.079 | 0.435 |
| AST | 0.266 | 0.007 |
| ALT | 0.135 | 0.181 |
| Alb | -0.498 | < 0.001 |
| PT | -0.435 | < 0.001 |
| Plt | -0.472 | < 0.001 |
| HA | 0.567 | < 0.001 |
| IV-c-7S | 0.428 | < 0.001 |

VT: Vascular trees; BMI: Body mass index; T-Bil: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; Alb: Albumin; PT: Prothrombin time; Plt: Platelet count; HA: Hyaluronic acid; IV-c-7S: Type IV collagen 7S.

correlation with Plt, Alb, and PT ($P < 0.01$) and a significant positive correlation with AST, HA, and IV-c-7S ($P < 0.01$). The SMI images and the CD34 expression at different stages of fibrosis are presented in Figure 4. The CD34 expression was mainly confined to the small vessels in the portal area and was also seen in the sinusoids in the liver parenchyma near the portal areas, with dotted, linear, semicircular, and circular staining patterns. In the mild fibrosis group (F0-1), CD34 staining was restricted to the endothelium of portal vessels. In contrast, numerous CD34-labeled vessels were detected in the advanced fibrosis group (F3-4). The VT significantly correlated with the CD34 LI ($r = 0.617$, $P < 0.001$) (Figure 5).

DISCUSSION

Doppler ultrasound is used to noninvasively measure blood flow velocity. To obtain high quality Doppler images, it is important to sufficiently suppress the clutter signals that originate from stationary and slowly moving tissue. The clutter signals overlap with the low velocity blood flow components. Clutter rejection filters are commonly used to remove the low frequency components in conventional Doppler imaging; however, these filters also cause the loss of signal from low velocity blood flow^[13,14]. SMI is an innovative ultrasound Doppler technique. It analyzes the clutter motion and uses a new adaptive algorithm to identify and remove tissue motion, revealing the true blood flow. SMI also features high frame rates (> 50 FPS) and high resolution. SMI operates in two modes: monochrome SMI (mSMI), which subtracts the background image from the detailed vasculature, and color SMI (cSMI), which displays the flow components in color overlaid on the grayscale B-mode image. SMI helps clinicians to visualize very small vascular structures and observe small branching details that previously were not visible. SMI

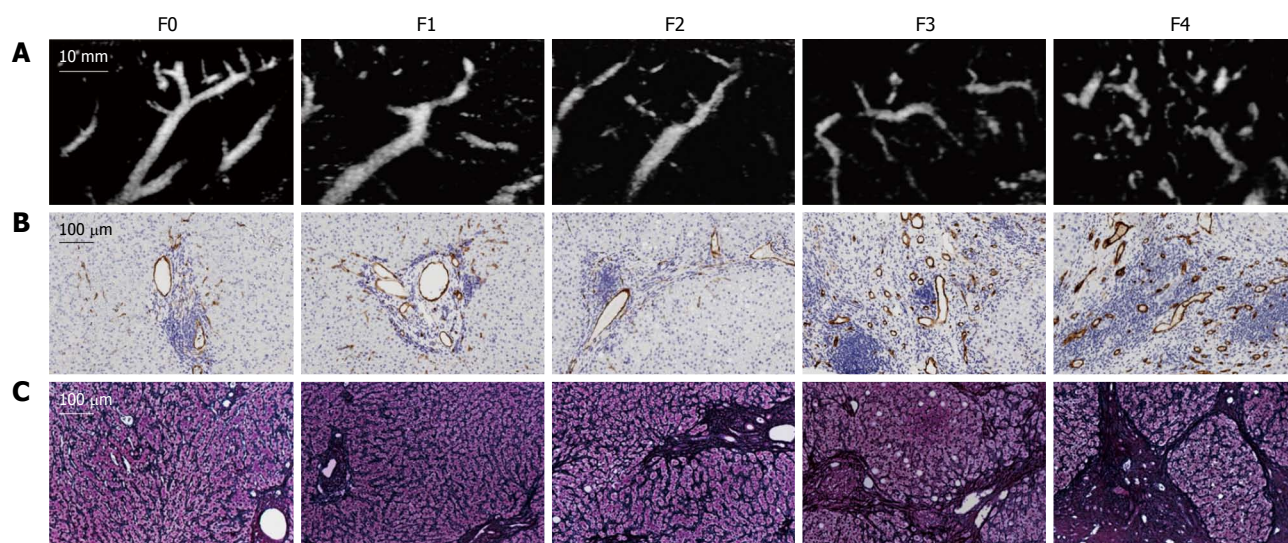


Figure 4 Superb microvascular imaging images and CD34 expressions at different fibrosis stages. A: SMI image; B: CD34 expression; C: Gomori trichrome staining. SMI: superb microvascular imaging.

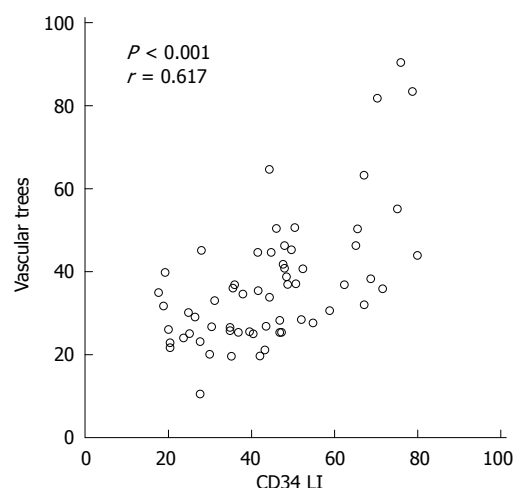


Figure 5 Correlation between the vascular trees value and the CD34 labeling index. Spearman's rank correlation coefficient. LI: Labeling index.

does not use intravenous contrast agents, which is a significant advantage for patients who are often fearful of needles and injections. Recently, several clinical studies have reported the use of SMI technology in performing microvascular evaluations. Ma *et al.*^[15] reported that SMI was more sensitive than color Doppler flow imaging for revealing the microvascular blood flow and vascularization of malignant breast tumors. According to their study, the detection of small vessels improved with the use of SMI compared with conventional Doppler ultrasound in malignant breast tumors. Moreover, Machado *et al.*^[16] reported that SMI consistently improved the depiction of thyroid microvascular flow in comparison to standard color and power Doppler imaging. To the best of our knowledge, the present study provides the first comparison of the vascular pattern on SMI and fibrosis staging in CLD patients.

The present study demonstrated a significant difference in the distribution of the SMI pattern and fibrosis stage, and the number of VT was significantly higher in the F4 stage than in the other stages. In addition, the AUROC curve for early prediction of the F4 stage by the VT was 0.911, indicating its high accuracy. The mechanism underlying the increase in the VT is thought to be derived from the combination of vessel branch grouping through the fibrous expansion of portal areas, angiogenesis that occurs with chronic liver damage, and fragmentation of the vessels that is caused by severe tortuosity. Sugimoto *et al.*^[21] reported such vascular tortuosity in advanced hepatic disease patients using CEUS. In fact, CEUS increases the detection of fine slow-velocity blood flow. However, the short arterial phase of the CEUS liver scan was not suitable for the present study.

An accurate evaluation of fibrosis in liver tissues is crucial for the differential diagnosis of CLD. A liver biopsy remains the reference standard for assessing liver fibrosis^[22,23]. However, this procedure is invasive and it is associated with patient discomfort, sampling error and, in rare cases, serious complications. Recent research has thus focused on the evaluation of noninvasive, valid, accurate, and flexible methods of assessing liver fibrosis. In recent years, transient elastography, acoustic radiation force impulse imaging, and shear wave elastography have been reported to indicate reliably the stage of liver fibrosis^[24-26]. However, ultrasonographic devices with these software programs are expensive high-end systems. In contrast, SMI is a noninvasive method that does not involve complicated operations and provides information that is superior to that obtained by color and power Doppler imaging. The results of this study indicate that SMI may be used for the early detection of advanced fibrosis.

The present study has shown that the VT is significantly correlated with PIt, AST, Alb, PT, HA, IV-c-7S, and the CD34 LI. In the present study, we performed an immunohistochemical analysis to evaluate the number of CD34-positive vessels. CD34 is preferentially expressed on the surface of regenerating or migrating endothelial cells and is a marker of proliferating endothelial cells in growth during angiogenesis. Our study is the first to show the number of SMI vascular signals to be correlated significantly with the CD34 expression.

There are several limitations associated with the present study. First, the study included a relatively small number of patients. A larger scale prospective clinical study is needed to quantify more accurately the optimal threshold of SMI for the diagnosis of fibrosis. Second, SMI is difficult to perform in obese individuals or patients with severe fatty liver due to the depth of subcutaneous fat; the increased distance between the deeper organs and the probe has a negative effect on the clarity of images of the hepatic vessels. Finally, the VT may depend on vessel fragmentation that occurs due to severe tortuosity. A method that allows for the three-dimensional examination of the hepatic vascular architecture is still needed.

In conclusion, SMI allowed the detailed delineation of the vascular architecture in CLD patients. Significant differences were found in the SMI pattern distribution and the fibrosis stage. The VT was significantly correlated with the expression of CD34. Thus, SMI appears to be a reliable tool for noninvasively detecting significant fibrosis or cirrhosis in patients with HCV. We now expect this imaging modality to continue to make further advances, including the development of three-dimensional SMI.

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COMMENTS

Background

Hepatitis C virus (HCV) has a high propensity to persist and cause chronic hepatitis, eventually leading to cirrhosis. Cirrhosis is an advanced stage of liver fibrosis that is accompanied by distortion of the hepatic vasculature. The evaluation of the hepatic vascular architecture is useful for assessing the HCV-related chronic liver disease (CLD) state, determining treatment strategies, and elucidating the mechanisms of disease progression. Therefore, the establishment of a noninvasive assessment tool of the hepatic vascular architecture is needed.

Research frontiers

Recently, Toshiba Medical Systems has developed a new Doppler technique called superb microvascular imaging (SMI). SMI is an innovative ultrasound Doppler technology employing a unique algorithm that allows visualization of minute vessels with slow velocity. However, no reports to date have assessed hepatic vascular architecture in patients with HCV-related CLD using SMI.

Innovations and breakthroughs

In this article, the authors validated that SMI allowed for the detailed delineation of the vascular architecture in CLD patients. Significant differences were found in the SMI pattern distribution and the fibrosis stage. Thus, SMI appears to be a reliable tool for noninvasively detecting significant fibrosis or cirrhosis in patients with HCV.

Applications

The number of SMI signals in the region of interest (number of vascular trees: VT) can therefore be used as a noninvasive biological marker for the early detection and quantitative evaluation of distortion of the hepatic vasculature.

Peer-review

The authors present valuable data from their research on the diagnosis of fibrosis using a new ultrasound Doppler technique called SMI in HCV patients.

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Retrospective Study

Impact of biliary stent-related events in patients diagnosed with advanced pancreaticobiliary tumours receiving palliative chemotherapy

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Abstract

AIM: To determine the impact (morbidity/mortality) of biliary stent-related events (SRE) (cholangitis or stent obstruction) in chemotherapy-treated pancreaticobiliary patients.

METHODS: All consecutive patients with advanced pancreaticobiliary cancer and a biliary stent *in-situ* prior to starting palliative chemotherapy were identified retrospectively from local electronic case-note records (Jan 13 to Jan 15). The primary end-point was SRE rate and the time-to-SRE (defined as time from first stenting before chemotherapy to date of SRE). Progression-free survival and overall survival were measured from the time of starting chemotherapy. Kaplan-Meier, Cox and Fine-Gray regression (univariate and multivariable) analyses were employed, as appropriate. For the analysis of time-to-SRE, death was considered as a competing event.

RESULTS: Ninety-six out of 693 screened patients were eligible; 89% had a metal stent (the remainder were plastic). The median time of follow-up was 9.6 mo (range 2.2 to 26.4). Forty-one patients (43%)

developed a SRE during follow-up [cholangitis (39%), stent obstruction (29%), both (32%)]. There were no significant differences in baseline characteristics between the SRE group and no-SRE groups. Recorded SRE-consequences were: none (37%), chemotherapy delay (24%), discontinuation (17%) and death (22%). The median time-to-SRE was 4.4 mo (95%CI: 3.6-5.5). Patients with severe comorbidities ($P < 0.001$) and patients with ≥ 2 baseline stents/biliary procedures [HR = 2.3 (95%CI: 1.2-4.44), $P = 0.010$] had a shorter time-to-SRE on multivariable analysis. Stage was an independent prognostic factor for overall survival ($P = 0.029$) in the multivariable analysis adjusted for primary tumour site, performance status and development of SRE (SRE group *vs* no-SRE group).

CONCLUSION: SREs are common and impact on patient's morbidity. Our results highlight the need for prospective studies exploring the role of prophylactic strategies to prevent/delay SREs.

Key words: Advanced biliary tract cancer; Pancreatic cancer; Biliary obstruction; Biliary stent; Stent-related event

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Core tip: Most patients diagnosed with advanced malignancies of the pancreas or bile ducts present with biliary obstruction; this requires biliary stenting before starting treatment with palliative chemotherapy. The impact of developing stent-related events (SRE) such as cholangitis or stent obstruction (and the potential role of prophylactic treatment in order to reduce the risk of developing SREs) has not been explored in this patient population. Our results have identified that SREs are common and adversely impact on patient's morbidity (and possibly mortality) and support the need for prospective studies investigating the role of prophylaxis in this population.

Lamarca A, Rigby C, McNamara MG, Hubner RA, Valle JW. Impact of biliary stent-related events in patients diagnosed with advanced pancreatobiliary tumours receiving palliative chemotherapy. *World J Gastroenterol* 2016; 22(26): 6065-6075 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i26/6065.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i26.6065>

INTRODUCTION

Both pancreatic and biliary tract malignancies are known to have a poor prognosis, mainly due to late presentation of patients who experience non-specific symptoms for some time. Because of this delay, the majority of patients (around 80%) are diagnosed with advanced-stage cancer, which is not amenable

to curative resection^[1,2]. In the context of advanced pancreatobiliary malignancies, chemotherapy is considered the standard of care treatment and cornerstone of patients management; while the role of radiotherapy is not clearly established (even for locally advanced disease), at least in the first-line setting. Chemotherapy is given with palliative intent, its aim being to increase survival and reduce cancer-related symptoms thereby improving quality of life. Systemic treatment for patients with advanced biliary tract cancer includes gemcitabine alone or given in combination with cisplatin^[3]. In patients with advanced pancreatic adenocarcinoma, chemotherapy may consist of monotherapy (gemcitabine) or combination therapy [gemcitabine-nab-paclitaxel doublet or FOLFIRINOX (5-fluorouracil, oxaliplatin and irinotecan)]^[4,5]. However, even with the newer chemotherapy combinations, the prognosis remains poor, with a median overall survival of less than 12 mo^[5].

For patients presenting with biliary obstruction, re-establishment of biliary drainage prior to starting palliative chemotherapy is mandatory [*via* endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography (PTC)]^[6]. Two main types of biliary stents are usually employed: (1) plastic stents which have a small diameter and are used for potentially-resectable tumours which are then removed when the curative surgery is performed; and (2) metallic stents that are usually chosen for patients with unresectable cancers because of their larger diameter^[7-9] and therefore, longer patency^[10].

Unfortunately, despite successful first biliary stenting, some patients will develop a stent-related event (SRE) such as recurrence of biliary obstruction (with development of new obstructive jaundice) or infection (cholangitis)^[11,12]. The median patency time of metallic biliary stents is estimated to be around 3.5 to 4.0 mo, although it varies depending on the diameter and type of the stent inserted (stent patency drops to 1.6 mo with plastic biliary stents)^[10,13,14]. The development of a SRE has been postulated to be detrimental in many ways for the patient population with pancreatobiliary cancer receiving palliative chemotherapy, leading to shorter survival (due to SRE-related life-threatening complications) and negative impact on patients' quality of life (due to repeat hospitalisation). Moreover, chemotherapy dose intensity may be compromised as a result of admission-related treatment delays or discontinuations (for example, in patients with permanent deterioration of their performance status after hospitalisation).

The aim of this study was to analyse the incidence (measured as SRE rate and time-to-SRE) and impact of SREs in patients with advanced biliary tract and pancreatic malignancies receiving palliative chemotherapy and, in doing so, to provide reference data in order to design an adequately-powered clinical trial to investigate the role of prophylaxis for the

prevention or delay of SREs in patients with biliary stents who are due to commence chemotherapy.

MATERIALS AND METHODS

Patients were identified retrospectively from local electronic case-note records at a single institution (The Christie NHS Foundation Trust, Manchester, United Kingdom). All consecutive patients diagnosed with hepato-pancreato-biliary (HPB) malignancies referred between January 2013 and January 2015 were screened. The local audit committee approved this study (CE15/1400).

Eligible patients were those meeting the following inclusion criteria: advanced (unresectable or metastatic) biliary tract malignancy [gallbladder, bile duct (cholangiocarcinoma) or ampullary] or pancreatic cancer (adenocarcinoma); had an *in-situ* biliary stent for biliary obstruction at the time of starting palliative chemotherapy; and went on to receive standard first-line palliative chemotherapy. Patients with hepatocellular carcinoma were excluded.

Demographic data [including fitness at baseline assessed by Eastern Cooperative Oncology Group Performance Status score (ECOG-PS)], characteristics of the primary tumour (tumour site and stage (AJCC 7th Edition)^[15]) and details of the treatment administered were collected from the local records. Radiological response to treatment was assessed 3-monthly as per Response Evaluation Criteria In Solid Tumours (RECIST v.1.1)^[16]. Comorbidities in addition to the index cancer were classified according to the Adult Comorbidity Evaluation (ACE)-27 index which is systematically used in our institution^[17]. Characteristics of the biliary stent fitted at baseline and details of any SRE (if any) were collected. Patients who developed at least one SRE during the follow-up were included in the SRE group, while those who did not were included in the no-SRE group.

The primary objective of this study was to assess the SRE rate and the time-to-SRE in a population of patients with a diagnosis of biliary or pancreatic cancer receiving palliative chemotherapy. Secondary objectives included analysis of the impact of the development of a SRE on the patient's planned chemotherapy schedule, progression-free survival (PFS) and overall survival (OS).

A stent-related event (SRE) was defined as any one or more of the following: (1) any episode of jaundice which was considered significant enough for new stenting or medical treatment and was confirmed by radiological imaging to be associated with biliary dilatation; (2) any episode of infection which was clinically in keeping with cholangitis (bile duct infection) requiring antibiotic therapy; (3) bacteraemia with isolation in blood cultures of bacteria suspected to have originated in the biliary tract; and (4) any episode of cholecystitis or gallbladder perforation.

The following were not considered SREs: (1)

jaundice related to high tumour burden liver disease with no significant change in biliary dilatation compared with previous imaging; (2) episodes of neutropenic or non-neutropenic fever with no identified biliary focus; and (3) patients with non-clinically significant biliary occlusion or biliary dilatation (*i.e.*, radiological evidence only with no jaundice, increasing bilirubin, increasing liver function tests (LFTs), fever or evidence of infection) who required no action (no new stenting or no new antibiotic therapy).

Time on follow-up was defined as the time from first biliary stent insertion to date of last follow-up available. Time-to-SRE was defined as the period between the date of the first biliary stenting and the date of the first evidence (clinical or radiological) of SRE. The median time-to-SRE was calculated in patients developing a SRE during follow-up. The risk of developing a SRE at different time-points was estimated for all patients, using the Kaplan-Meier method. For the analysis of time-to-SRE, death was considered a competing event; thus, Fine-Gray regression was employed for identification of factors related to longer/shorter time-to-SRE. For multivariable analysis of factors impacting time-to-SRE, those variables which showed statistically significant *P*-value in the univariate analysis (*P* < 0.05) were included.

In order to provide data regarding the impact of chemotherapy in PFS and OS, PFS and OS were defined as the time from starting chemotherapy to the time of progression (radiological or clinical) and the date of death/last follow-up, respectively. Median PFS and OS were estimated by the Kaplan-Meier method. The log-rank test and univariate/multivariable Cox regression models were used to identify potential prognostic factors for both PFS and OS. For assessment of factors with an impact on OS, variables considered of interest [such as site of primary tumour, stage, ECOG-PS and development of SRE (SRE group vs no-SRE group)] and those variables which showed statistically significant *P* in the univariate analysis (*P* < 0.05) were included in multivariable analysis.

Statistical *t*-test, χ^2 test and the Mann-Whitney test (in case of non-normal distribution as per Shapiro-Wilk test) were applied as appropriate. Two-sided significance test with a *P* of < 0.05 was considered significant. Stata version 12.0 software was employed for the statistical analysis.

RESULTS

A total of 693 patients diagnosed with HPB malignancies were screened; 96 met the criteria for inclusion (Figure 1). The median time of follow-up was 9.6 mo (range 2.2 to 26.4). By the end of the follow-up period, 45% and 69% of the patients had progressed and died, respectively. There were no significant differences (*P* = 0.1308) in median follow-up between the SRE group [10.5 mo (range: 2.1-26.4)] and the no-SRE group [8.5 mo (range 3.2-18.9)].

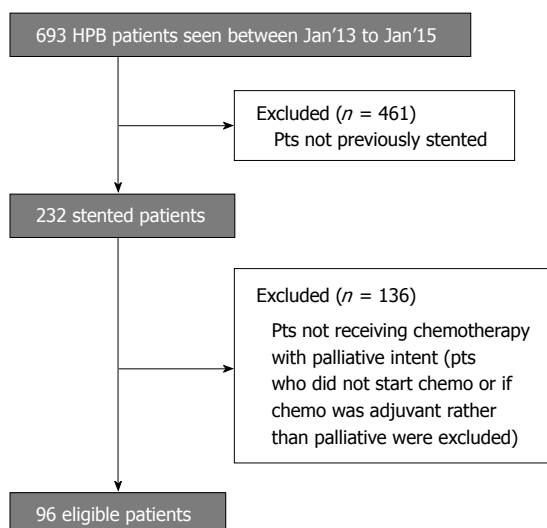


Figure 1 Patient flow. Ninety-six out of the 693 patients screened were found to be eligible. HPB: Hepato-pancreato-biliary cancer; Jan: January; Pts: Patients.

The rate of patients who died and progressed was also similar between both groups [rate of death: 71% (SRE group) vs 67% (no-SRE group); $P = 0.825$] [rate of progression: 54% (SRE group) vs 38% (no-SRE group); $P = 0.151$].

Patient demographics

The median age at the time of commencing palliative chemotherapy was 66.6 years (range 26-83.8) with a similar proportion of males (56%) and females (44%). The primary tumour site was as follows: 78% pancreas, 18% bile duct (cholangiocarcinoma), 3% ampulla and 1% gallbladder cancer. Most patients (60%) had locally advanced disease. All patients were fit for chemotherapy and started first-line systemic treatment as per clinician discretion. The median time between first stenting and start of chemotherapy was 1.8 mo (range: 0.1-12.6). The most frequently used chemotherapy schedules were single agent gemcitabine (39%) followed by gemcitabine and capecitabine combination (26%). The median time of chemotherapy duration was 3.2 mo (range 0.1-7.6); there were no differences in baseline characteristics between the SRE group and the no-SRE group (see detail in Table 1). None of the patients included were on long-term antibiotics or ursodeoxycholic acid.

Biliary stenting characteristics at baseline

Most of patients had one (73%) or two (22%) biliary stents fitted at the time of starting systemic chemotherapy; only 3 patients and 1 patient had three and four stents *in-situ*, respectively. In 85 patients (89%), stents were metallic. A higher proportion of patients in the SRE group when compared to the no-SRE group had ≥ 2 biliary stents or biliary procedures [41% (SRE group) vs 15% (no-SRE group); $P = 0.004$]. See Table 2.

Stent-related events rate and its consequences

During follow-up, 41 (43%) patients developed a SRE; the type of SRE was as follows: cholangitis (16 patients; 39%), stent obstruction (12; 29%) and combination of both (13; 32%). Moreover, in 14 out of the 41 patients with a first SRE (34%), further SREs were documented. Development of at least one SRE during the follow-up led to the following consequences: a delay in chemotherapy (10 patients; 24%), interruption of chemotherapy (7; 17%) and death (9; 22%). In 15 of the patients (37%), there was no significant SRE-related repercussion. No relationship was found between type of stent and type of SRE ($P = 0.815$; full data not show); nor between the type of SRE and its consequence ($P = 0.166$; full data not shown). See Table 2.

Time to stent-related event

The median time-to-SRE was 4.4 mo (95%CI: 3.6-5.5) when calculated for the SRE group only. Table 3 summarises the estimated risk of SRE for all patients (SRE group and no-SRE group) at different time-points during the follow up, showing a cumulative risk of developing SRE during the time on follow-up. Figure 2 represents each of the patients included in this study, showing the time to SRE in the context of other clinically significant events.

Patients with severe comorbidities (vs patients with no comorbidities) ($P < 0.001$) and patients with ≥ 2 stent/biliary procedure before starting chemotherapy (vs 1) had shorter time-to-SRE on multivariable analysis (HR = 2.3, 95%CI: 1.2-4.44, $P = 0.010$). See Table 4.

Progression-free survival

Only nine patients (9%) were still receiving first-line chemotherapy at the time of the analysis: eight in the no-SRE group and one in the SRE group. The most frequent reason for stopping chemotherapy was toxicity (46%), followed by completion of planned treatment (27%), progressive disease (17%) or death (1%). Estimated median PFS was 6.7 mo (95%CI: 4.4-7.8), with similar results in both SRE group and no-SRE group [6.7 (95%CI: 4.3-8.7) and 6.8 (95%CI: 3.9-7.8), respectively] [HR = 0.9 (95%CI: 0.6-1.5), $P = 0.7666$]. There were no statistically significant differences with respect to the reason for chemotherapy discontinuation between the SRE group and the no-SRE group ($P = 0.058$; full data not shown).

Overall survival

The estimated median OS was 8.6 mo (95%CI: 6.8-9.8). Even though there seemed to be a trend for longer survival in the SRE group [median OS 9.8 mo (95%CI: 7.4-11.6)] than in the no-SRE group [median OS 7.6 mo (95%CI: 5.7-9.6)] differences were not statistically significant (Log-rank test $P =$

Table 1 Demographic characteristics of patients included in the study

| Variables | | All patients (n = 96) | SRE group (n = 41; 43%) | no-SRE group (n = 55; 57%) | P-value for distribution within baseline parameter (χ^2 test), SRE vs no-SRE groups |
|---|--------------------------------|-----------------------|-------------------------|----------------------------|---|
| Gender | Female | 42 (44) | 20 (49) | 22 (40) | 0.391 |
| | Male | 54 (56) | 21 (51) | 33 (60) | |
| Age ¹ | Median (range) | 66.6 (26-83.8) | 64.9 (26-84) | 67.6 (42.4-83.2) | 0.8833 ² |
| Primary tumour site | Ampulla | 3 (3) | 1 (2) | 2 (4) | 0.380 ³ |
| | Bile duct (cholangiocarcinoma) | 17 (18) | 10 (24) | 7 (13) | |
| | Intrahepatic | 5 (31) | 3 (33) | 2 (29) | |
| | Extrahepatic | 11 (69) | 6 (67) | 5 (71) | 1.000 ⁴ |
| | Gallbladder | 1 (1) | 0 (0) | 1 (2) | |
| | Pancreas | 75 (78) | 30 (73) | 45 (82) | 1.000 ⁵ |
| | Head | 66 (89) | 26 (90) | 40 (89) | |
| Stage | Body | 8 (11) | 3 (10) | 5 (11) | 0.294 |
| | Locally advanced | 58 (60) | 22 (54) | 36 (65) | |
| ECOG-PS | Metastatic | 38 (40) | 19 (46) | 19 (35) | 0.547 |
| | 0 | 17 (18) | 9 (22) | 8 (15) | |
| Diabetic | 1 | 51 (53) | 22 (54) | 29 (53) | 1.000 |
| | ≥ 2 | 28 (29) | 10 (24) | 18 (33) | |
| | No | 68 (71) | 29 (71) | 39 (71) | |
| Comorbidities | Yes | 28 (29) | 12 (29) | 16 (29) | 0.428 |
| | None | 31 (32) | 14 (34) | 17 (31) | |
| | Mild | 41 (43) | 18 (44) | 23 (42) | |
| | Moderate | 20 (21) | 9 (22) | 11 (20) | |
| Line of palliative chemotherapy | Severe | 4 (4) | 0 (0) | 4 (7) | 1.000 |
| | First | 96 (100) | 41 (100) | 55 (100) | |
| Type of chemotherapy | FOLFIRINOX | 11 (11) | 4 (10) | 7 (13) | 0.605 |
| | Cisplatin Gemcitabine | 13 (14) | 8 (20) | 5 (9) | |
| | Gemcitabine Nab-paclitaxel | 7 (7) | 2 (5) | 5 (9) | |
| | Gemcitabine +/- TH302 | 2 (2) | 0 (0) | 2 (4) | |
| | Gemcitabine Capecitabine | 25 (26) | 12 (29) | 13 (24) | |
| | Gemcitabine single agent | 37 (39) | 15 (37) | 22 (40) | |
| | FOLFOX | 1 (1) | 0 (0) | 1 (2) | |
| Time from first stent to starting chemotherapy ¹ | Median (range) | 1.8 (0.1-12.6) | 1.6 (0.6-5.8) | 1.9 (0.1-12.6) | 0.1824 ² |
| Time of chemotherapy duration ¹ | Median (range) | 3.2 (0.1-7.6) | 3.8 (0.1-7.2) | 3.1 (0.1-7.6) | 0.4520 ² |

No differences were identified between SRE group and the no-SRE group. ¹Variables do not meet a normal distribution (as per Shapiro-Wilks test); ²Mann-Whitney *P*-value has been provided for variables not meeting normal distribution criteria; ³the *P* for χ^2 test for comparison of distribution of primary tumour [ampulla *vs* bile duct (cholangiocarcinoma) *vs* gallbladder *vs* pancreas] between SRE group and no-SRE group; ⁴the *P* for χ^2 test for comparison of distribution of primary tumour [type of bile duct tumour (cholangiocarcinoma): intrahepatic *vs* extrahepatic] between SRE group and no-SRE group; ⁵The *P* for χ^2 test for comparison of distribution of primary tumour (site of pancreatic cancer: head *vs* body) between SRE group and no-SRE group. SRE: Stent-related event; ECOG-PS: ECOG performance status; FOLFIRINOX: 5-fluorouracil, oxaliplatin and irinotecan combination; FOLFOX: 5-fluorouracil and oxaliplatin combination.

0.0947). When the impact on OS of the SRE-related consequence was analysed, we identified a longer OS in the group of patients with mild consequences [none/chemotherapy delay; median OS 11.6 mo (95%CI: 9.8-20)] compared to those with severe consequences [interruption of chemotherapy or death; median OS 4.4 mo (95%CI: 2.6-8.7)]; [HR = 3.8 (95%CI: 1.7-8.2), *P* = 0.001] (Figure 3). Stage was an independent prognostic factor for OS [HR = 1.8 (95%CI: 1.06-2.9), *P* = 0.029] in multivariable analysis adjusted for primary tumour, ECOG-PS and development of SRE (SRE group *vs* no-SRE group) (Table 5).

DISCUSSION

In patients with advanced/inoperable cancers of the pancreas or biliary tract receiving chemotherapy and with an indwelling biliary stent at the start of treatment, we observed a high rate of SREs; moreover two-thirds of patients had some kind of consequence from the SRE (chemotherapy delay, discontinuation or early death). In addition, one-third of patients with a first SRE developed further events, highlighting the importance of close follow-up for early detection and management of such events. Although we observed

Table 2 Characteristics of the baseline biliary stenting and stent-related event

| Variables | | All patients (n = 96) | SRE group (n = 41; 43%) | no-SRE group (n = 55; 57%) | P-value for distribution within baseline parameter (χ^2 test), SRE vs no-SRE groups |
|---|---------------------------------|--------------------------|-------------------------------|-------------------------------|---|
| Stents at baseline | 1 | 70 (73) | 24 (59) | 46 (84) | 0.008 |
| | 2 | 21 (22) | 13 (32) | 8 (14) | |
| | 3 | 3 (3) | 3 (7) | 0 (0) | |
| | 4 | 1 (1) | 1 (2) | 0 (0) | |
| | Not specified | 1 (1) | 0 (0) | 1 (2) | |
| Number of stents/biliary interventions at baseline | 1 previous stent/intervention | 70 (73) | 24 (59) | 46 (84) | 0.004 |
| | ≥ 2 previous stent/intervention | 25 (26) | 17 (41) | 8 (15) | |
| | Not specified | 1 (1) | 0 (0) | 1 (1) | |
| Type of stent (baseline) | Metal | 85 (89) | 37 (90) | 48 (87) | 0.170 |
| | Plastic | 7 (7) | 4 (10) | 3 (5) | |
| | Not specified | 4 (4) | 0 (0) | 4 (7) | |
| Type of SRE (SRE group only) | Cholangitis | 16 (17) | 16 (39) | - | - |
| | Stent obstruction | 12 (13) | 12 (29) | - | |
| | Both | 13 (14) | 13 (32) | - | |
| Consequence of SRE (SRE group only) | None | 15 (16) | 15 (37) | - | - |
| | Chemotherapy delayed | 10 (10) | 10 (24) | - | |
| | Chemotherapy stopped | 7 (7) | 7 (17) | - | |
| | Death | 9 (9) | 9 (22) | - | |
| Further SRE (SRE group only) | No | 27 (28) | 27 (66) | - | - |
| | Yes | 14 (15) | 14 (34) | - | |

Forty-three percent of patients developed a SRE during the follow-up. SRE: Stent-related event.

Table 3 Risk of development of stent-related event increased with longer follow-up period in the absence of competing event (death)

| Time-point of follow-up since first biliary stenting | Estimated risk of development of SRE rate for all patients |
|---|---|
| 3 mo | 11.5% (95%CI: 6.5-19.7) |
| 6 mo | 32.0% (95%CI: 23.5-42.7) |
| 12 mo | 48.6% (95%CI: 37.5-61) |
| 18 mo | 59.9% (95%CI: 44-76.5) |
| 24 mo | 79.9% (95%CI: 48.03-98.1) |

SRE: Stent-related event.

no significant relationship between the type of stent and type of SRE, this may be explained by the small proportion of patients (11%) with plastic stents. Finally, there were no differences between the type of SRE developed (obstruction, infection or both) and its consequences; be it mortality, chemotherapy delay or discontinuation rate. Therefore all SREs should be considered as a medical emergency and early management is essential, due to the potentially life-threatening consequences.

Stent-related events occurred early with a median time-to-SRE of only 4.4 mo. Moreover, the risk increases with time rising 3-fold between month 3 and month 6 and up to 80% in patients alive at 24 mo. This highlights the importance of clinician (including primary and secondary care) and patient (and their cares) awareness of early detection and treatment of a SRE. Although some guidelines suggest replacement of plastic stents every six months^[18] there are no such recommendations for metallic stents.

The only factor associated with a higher rate of

SRE was the number of biliary stents or procedures at baseline (1 vs ≥ 2); none of the other baseline characteristics had this impact, including disease stage or site of primary tumour, highlighting the challenge that clinicians face in identifying patients at increased risk of a SRE. In addition to the number of stents at baseline, the presence of severe comorbidity was associated with earlier development of a SRE (*i.e.*, earlier time-to-SRE). The fact that stage had no impact on time-to-SRE is likely to reflect the fact that stent occlusion arises from the primary (stented) disease rather than metastases, in the vast majority of patients.

The development of a SRE may be expected to be more frequent in patients receiving chemotherapy due to its known myelosuppressive effect^[13] and particularly in patients receiving highly myelosuppressive treatment, such as FOLFIRINOX^[5]. This was not confirmed in this study although this may again be due to the small number of patients receiving this regimen, and the fact that prophylactic granulocyte-colony stimulating factor (G-CSF) was routinely prescribed for these patients to reduce duration of neutropenia. The median time-to-SRE in our study was similar to previously published data in a non-chemotherapy population^[13], suggesting that chemotherapy may not have as much as an impact on SREs as might be expected. Neither did we observe a higher rate of SREs if chemotherapy was delayed at baseline (due to potentially greater risk of tumour in-growth).

The development of a SRE did not impact on PFS; however there was a non-significant trend towards longer OS in the SRE group, compared with the no-SRE group. This cannot be interpreted as a causality effect

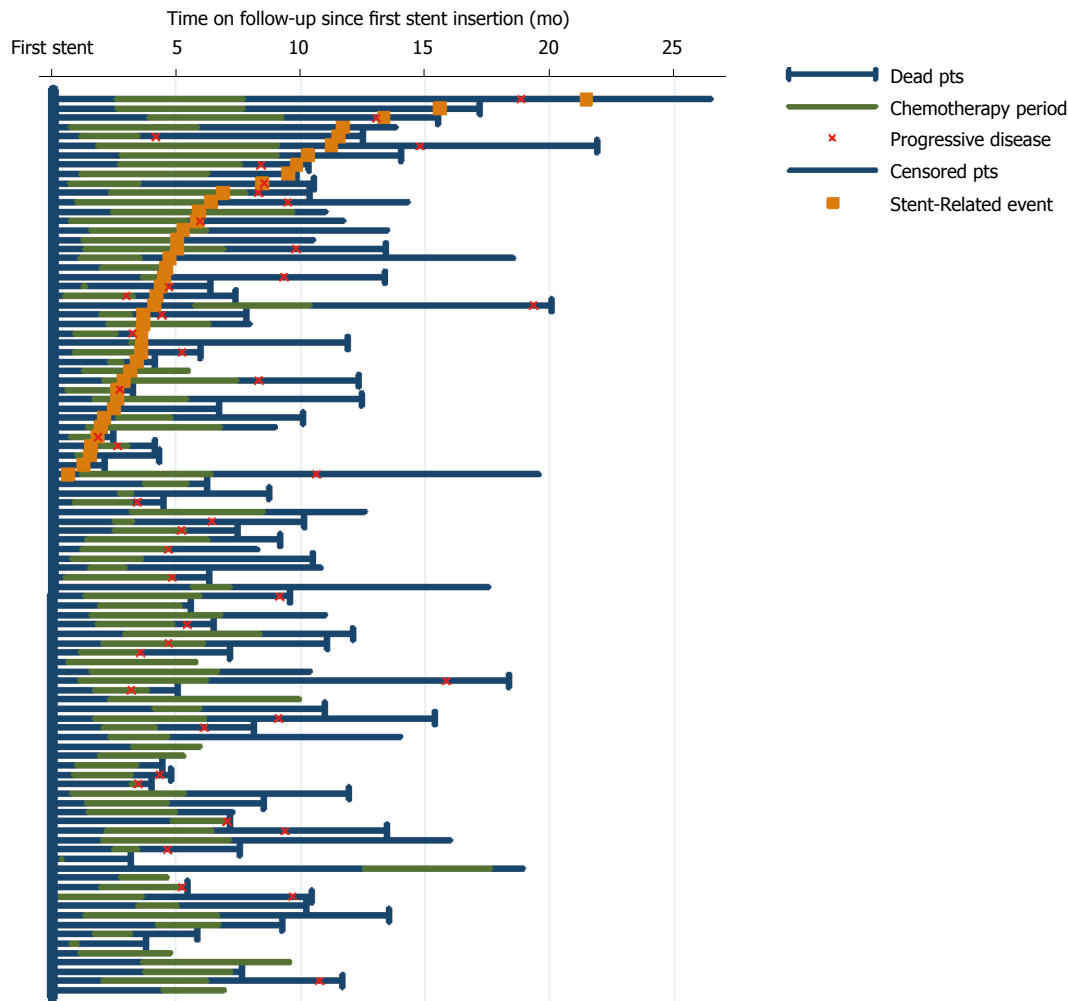


Figure 2 Graphical representation of each of the patient's follow-up included in this study, time on chemotherapy, and time of radiological progression and development of a stent-related event.

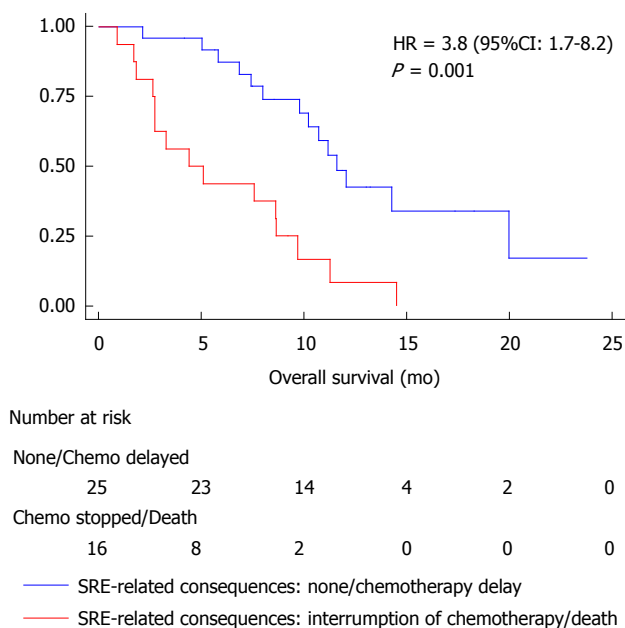


Figure 3 Kaplan Meier graphic. Overall survival and type of stent-related event (SRE)-related consequence [mild (none/chemotherapy delayed) vs severe (chemotherapy interrupted/death)].

(patients with SRE live longer) but rather a “time-at-risk” effect (patients who live longer have more time to develop a SRE). This was the main reason for including death as a “competing event” in the statistical analysis for time-to-SRE. In fact, the development of a SRE did not impact on survival in the multivariable analysis for OS, confirming this approach. Importance of “time-at-risk” in the development of a SRE is also supported by the following: a higher number of patients still receiving chemotherapy at the data cut-off point in the no-SRE group (8 patients vs 1 patient in the SRE group); and longer (though statistically non-significant) follow-up in the SRE group.

Our study population is representative of the population of interest when comparing characteristics such as rate of locally advanced patients; higher rate of biliary obstruction in patients with locally advanced disease^[18]; predominance of pancreatic cancer compared to biliary malignancies^[19]; median PFS and median OS (in keeping with a non-trial population). Moreover, the fact that tumour stage was identified as a prognostic factor in the multivariable analysis for OS was reassuring. The majority of patients with metallic

Table 4 Univariate and multivariable analysis looking for factors related with time-to-stent-related event

| Time-to-SRE | | Univariate analysis (Fine-Gray regression) | | Multivariable analysis (Fine-Gray regression) | |
|--|------------------|--|---------|--|---------|
| | | HR (95%CI) | P-value | HR (95%CI) | P-value |
| Primary | BTC | Ref | | X | |
| | Pancreas | 0.8 (0.4-1.5) | 0.407 | | |
| Stage | Locally advanced | Ref | | X | |
| | Metastatic | 1.4 (0.8-2.6) | 0.251 | | |
| ECOG-PS | 0/1 | Ref | | X | |
| | ≥ 2 | 0.8 (0.4-1.5) | 0.435 | | |
| Comorbidities | None | Ref | | Ref | |
| | Mild | 0.8 (0.4-1.7) | 0.605 | 1.1 (0.5-2.2) | 0.844 |
| | Moderate | 0.9 (0.4-1.9) | 0.734 | 1.1 (0.5-2.2) | 0.986 |
| | Severe | 3.6×10^{-8} (1.2×10^{-8} - 1.1×10^{-7}) | < 0.001 | 9.4×10^{-7} (2.9×10^{-7} - 3.1×10^{-6}) | < 0.001 |
| Number of stents/biliary interventions at baseline | 1 | Ref | | Ref | |
| | ≥ 2 | 2.5 (1.4-4.6) | 0.003 | 2.3 (1.2-4.44) | 0.010 |
| Type of the most recent stent | Metal | Ref | | X | |
| | Plastic | 2.1 (0.7-6.5) | 0.182 | | |

Fine-Gray Regression; competing event: Death. BTC: Biliary tract cancer; ECOG-PS: ECOG performance status.

Table 5 Univariate and multivariable analysis looking for factors related with overall survival (Cox Regression)

| Overall survival | | Univariate analysis (Cox regression) | | Multivariable analysis (Cox regression) | |
|---------------------|------------------|--------------------------------------|---------|---|---------|
| | | HR (95%CI) | P-value | HR (95%CI) | P-value |
| Primary site | BTC | Ref | | Ref | |
| | Pancreas | 1.6 (0.8-2.9) | 0.153 | 1.5 (0.8-2.8) | 0.205 |
| Stage | Locally advanced | Ref | | Ref | |
| | Metastatic | 1.6 (0.99-2.9) | 0.067 | 1.8 (1.06-2.9) | 0.029 |
| ECOG-PS | 0/1 | Ref | | Ref | |
| | ≥ 2 | 0.9 (0.5-1.6) | 0.748 | 0.9 (0.5-1.6) | 0.716 |
| Stent-related event | No-SRE group | Ref | | Ref | |
| | SRE group | 0.7 (0.4-1.1) | 0.098 | 0.6 (0.4-1.01) | 0.205 |

For assessment of factors with an impact on OS, variables considered of interest [such as site of primary tumour, stage, ECOG-PS and development of SRE (yes/no)] and those variables which showed statistically significant *P*-value in the univariate analysis were included in multivariable analysis. BTC: Biliary tract cancer; ECOG-PS: ECOG performance status.

stents at baseline is in keeping with international standards for palliative patients who are expected to be treated with chemotherapy (*i.e.*, have an estimated survival of > 3 mo) in whom a plastic stent should not be considered as a standard^[20]. The small number of patients with ECOG ≥ 2 is the likely reason why ECOG-PS did not impact on OS as chemotherapy is usually considered only for patients of good performance status (PS 0-1 and selected PS 2 patients).

There are limitations associated with our retrospective series; although all consecutive patients with a diagnosis of advanced pancreaticobiliary malignancy were included, the patients were already pre-selected by fitness and comorbidities for referral for consideration for chemotherapy. Moreover, retrospective collection of data may be subject to reporting bias. In addition, patients with different primary tumour sites were included who were in receipt of differing chemotherapeutic agents; however completeness of data and inclusion of patients from a recent era makes our findings credible. Our series did not include any non-stented patients and therefore comparisons of SRE rate between stented

and not-stented populations, which could be useful for assessing whether the combination of chemotherapy and biliary stent increased the risk of SRE, are not possible. Finally, most of our patients had a metal stent *in situ*; making our data not representative of population with plastic biliary stents.

To date, there is no evidence supporting the use of prophylactic therapy, such as antibiotics or ursodeoxycholic acid, aimed at reducing or delaying SREs in these patients; thus clinicians currently treat rather than prevent SREs^[33]. One purpose of our study was to generate data to inform the design of future clinical trials exploring the role of prophylaxis for the prevention or delay of SREs in this specific population. This rationale has already been investigated by some studies (summarised in Table 6): overall, these trials are under-powered and involved patients with both benign and malignant biliary strictures who had plastic stents *in-situ*. No adequately-powered studies have been performed; neither has this question been addressed in patients with metal stents (now considered the standard of care in the palliative setting) or in a population receiving chemotherapy

Table 6 Summary of the available literature exploring the role of prophylactic treatment for stent-related event

| Disease | Ref. | Randomised | Type of stent | Total number of patients | Number of patients per arm | Treatment arm(s): Stent insertion plus.... | Investigation and result |
|-----------|---|----------------------|---------------|----------------------------|--|--|---|
| Benign | Sciumè <i>et al</i> ^[21] , 2004 | Yes (not blinded) | Plastic | 90 | 49/41 | Ursodeoxycholic acid and levofloxacin <i>vs</i> Ursodeoxycholic acid alone | Longer stent patency with lower cholangitis and admission rate. |
| | Katsinelos <i>et al</i> ^[22] , 2008 | Yes (blinded) | Plastic | 41 | 21/20 | Ursodeoxycholic acid <i>vs</i> Placebo | Common bile duct stones. No reduction in the bile duct stone size. |
| | Han <i>et al</i> ^[23] , 2009 | No | Plastic | 28 | 28 | Ursodeoxycholic acid and terpene | Gallstones in elderly patients. Size of gallstones was reduced. |
| | Lee <i>et al</i> ^[24] , 2011 | No | Plastic | 51 | 51 | Ursodeoxycholic acid | Gallstones in elderly patients. No benefit of adding Ursodeoxycholic acid. |
| | Nishizawa <i>et al</i> ^[25] , 2013 | No | Plastic | 36 patients, 63 procedures | Non-randomised, two arms: 20/43 procedures | Ursodeoxycholic acid <i>vs</i> Observation | Bile duct stones. Longer patency time and reduction in gallstone size in the intervention cohort. |
| Malignant | Ghosh <i>et al</i> ^[26] , 1994 ¹ | Yes (not blinded) | Plastic | 70 | 31/39 | Ursodeoxycholic acid + antibiotic (ampicillin, metronidazole, ciprofloxacin) <i>vs</i> Observation | No differences in stent occlusion rate. |
| | Barrioz <i>et al</i> ^[27] , 1994 ¹ | Yes (not blinded) | Plastic | 20 | Not specified | Ursodeoxycholic acid and norfloxacin <i>vs</i> Observation | Longer stent patency, prolonged median survival and shorter mean hospital stay. |
| | Luman <i>et al</i> ^[28] , 1999 ¹ | Yes (not blinded) | Not specified | 40 | 20/20 | Ciprofloxacin and rowachol <i>vs</i> Observation | Similar rate of obstruction and time to event. |
| | Sung <i>et al</i> ^[29] , 1999 ¹ | Yes (not blinded) | Plastic | 58 | Not specified | Ursodeoxycholic acid <i>vs</i> Observation | Similar rate of obstruction and time to event. |
| | De Lédinghen <i>et al</i> ^[30] , 2000 ¹ | Yes (not blinded) | Plastic | 62 | 33/29 | Ursodeoxycholic acid and norfloxacin <i>vs</i> Observation | Stopped after the interim analysis. No differences in stent patency. |
| | Halm <i>et al</i> ^[31] , 2001 ² | Yes (not blinded) | Plastic | 52 | 26/26 | Ursodeoxycholic acid and ofloxacin <i>vs</i> Ursodeoxycholic acid alone | Similar rate of obstruction and times to stent obstruction. |
| | Chan <i>et al</i> ^[32] , 2005 ² | Yes (double blinded) | Plastic | 94 | 50/44 | Ciprofloxacin <i>vs</i> Placebo | No differences in stent patency. Lower rate of cholangitis, but there was improvement in quality of life. |

Overall, studies are underpowered for reaching definitive conclusions. ¹These studies were included in The Cochrane review^[33]; ²These studies were not included in The Cochrane review^[33].

for advanced pancreas/biliary cancer. In 2002 the Cochrane collaboration concluded that well-designed studies with sufficient statistical power were essential to address this issue^[33]. Our results highlight the importance of performing adequately-powered prospective studies looking for prevention of these events.

Stent-related events can result in life-threatening complications in patients with advanced pancreaticobiliary cancer who are receiving palliative chemotherapy; 43% of patients in our series developed a SRE and 63% of them had a SRE-related impact on delivery of chemotherapy or resulting in death. The risk of developing SREs increases with prolonged time on treatment and/or follow-up; moreover, risk is higher in

patients with severe comorbidities and patients with ≥ 2 biliary stent or biliary procedures at baseline. Thus, close monitoring for early diagnosis and treatment is required. Our data will inform the design of future, prospective clinical trial(s) to evaluate how the risk of SREs and their sequelae can be reduced; as well as the clinical and socio-economic impact of doing so.

COMMENTS

Background

Despite successful first biliary stenting, some patients with biliary and pancreatic malignancies will develop a stent-related event (SRE) such as recurrence of biliary obstruction (with development of new obstructive jaundice) or infection (cholangitis). Development of these events is detrimental, especially

in a chemotherapy-treated population.

Research frontiers

The aim of this study was to analyse the incidence (measured as SRE rate and time-to-SRE) and impact of SREs in patients with advanced biliary tract and pancreatic malignancies receiving palliative chemotherapy and, in doing so, to provide reference data in order to design an adequately-powered clinical trial to investigate the role of prophylaxis for the prevention or delay of SREs in patients with biliary stents who are due to commence chemotherapy.

Innovations and breakthroughs

In patients with advanced/inoperable cancers of the pancreas or biliary tract receiving chemotherapy and with an indwelling biliary stent at the start of treatment, the authors observed a high rate of SREs; moreover, in two-thirds of these patients there was a direct consequence from the SRE (chemotherapy delay, discontinuation or early death). Therefore all SREs should be considered as a medical emergency and early management is essential, due to their potentially life-threatening consequences.

Applications

Although the authors have demonstrated that SREs are frequent and may be associated with adverse outcomes, there is, to date, no evidence supporting the use of prophylactic therapy, such as antibiotics or ursodeoxycholic acid, aimed at reducing or delaying SREs in these patients; thus clinicians currently treat rather than prevent SREs. One purpose of our study was to generate data to inform the design of future clinical trials exploring the role of prophylaxis for the prevention or delay of SREs in this specific population.

Terminology

Stent-related events: recurrence of biliary obstruction with stent obstruction (with development of new obstructive jaundice) or infection (cholangitis) following successful first biliary stenting.

Peer-review

The authors explored the occurrence and consequences of stent-related events in a retrospective cohort of patients with pancreatico-biliary cancer stented for biliary obstruction. They showed that 43% patients developed a stent-related during the follow-up, which could lead to chemotherapy delay or discontinuation, or death.

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Retrospective Study

Preoperative defining system for pancreatic head cancer considering surgical resection

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Abstract

AIM: To provide appropriate treatment, it is crucial to share the clinical status of pancreas head cancer among multidisciplinary treatment members.

METHODS: A retrospective analysis of the medical records of 113 patients who underwent surgery for pancreas head cancer from January 2008 to December 2012 was performed. We developed preoperative defining system of pancreatic head cancer by describing "resectability - tumor location - vascular relationship - adjacent organ involvement - preoperative CA19-9 (initial bilirubin level) - vascular anomaly". The oncologic correlations with this reporting system were evaluated.

RESULTS: Among 113 patients, there were 75 patients (66.4%) with resectable, 34 patients (30.1%) with borderline resectable, and 4 patients (3.5%) with locally advanced pancreatic cancer. Mean disease-free survival was 24.8 mo (95%CI: 19.6-30.1) with a 5-year disease-free survival rate of 13.5%. Pretreatment tumor size ≥ 2.4 cm [Exp(B) = 3.608, 95%CI: 1.512-8.609, $P = 0.044$] and radiologic vascular invasion [Exp(B) = 5.553, 95%CI: 2.269-14.589, $P = 0.002$] were independent predictive factors for neoadjuvant treatment. Borderline resectability [Exp(B) = 0.222, $P = 0.008$], pancreatic

head cancer involving the pancreatic neck [Exp(B) = 9.461, $P = 0.001$] and arterial invasion [Exp(B) = 6.208, $P = 0.010$], and adjusted CA19-9 ≥ 50 [Exp(B) = 1.972, $P = 0.019$] were identified as prognostic clinical factors to predict tumor recurrence.

CONCLUSION: The suggested preoperative defining system can help with designing treatment plans and also predict oncologic outcomes.

Key words: Preoperative defining system; Pancreas head cancer; Borderline resectable; Adjusted CA19-9; Neoadjuvant therapy

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Core tip: Owing to the anatomical complexity of the pancreas head cancer, it is not always easy to share the exact disease status among multidisciplinary treatment members. So, we made a preoperative defining system, which contained the important clinical variables (resectability, tumor location, vascular relationship, adjacent organ involvement, preoperative CA19-9, vascular anomaly) to decide the treatment plan for pancreas head cancer. Through internal validation, we proved that this system could be useful not only to clarify the disease characteristics but also to predict oncologic outcomes of pancreas head cancer.

Yang SJ, Hwang HK, Kang CM, Lee WJ. Preoperative defining system for pancreatic head cancer considering surgical resection. *World J Gastroenterol* 2016; 22(26): 6076-6082 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i26/6076.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i26.6076>

INTRODUCTION

Pancreatic cancer is one of the lethal malignant diseases arising from the gastrointestinal tract. It is well-known that only margin-negative resection of the tumor can lead to long-term survival^[1]. However, most patients treated with curative pancreatectomy develop tumor recurrences, especially in the liver. Therefore, effective adjuvant systemic chemotherapy should be mandatory^[2].

In general, resectable pancreatic cancer is defined as a clinical tumor condition confined to the pancreas without radiologic evidence suggesting invasion of the major vascular system or systemic metastases. However, there is some controversy about the definition of borderline resectable pancreatic cancer^[3]. Currently, there are two systems for defining borderline resectable pancreatic cancer^[4,5]. Whatever definition is chosen, surgeons should plan the treatment modality and design the operative approach for curative resection based on preoperative radiologic assessment. In addition, they need to explain the

patients' chance of survival and prognosis based on clinically available information. However, there is no generalized preoperative defining system effectively showing the extent of pancreatic cancer and tumor biology.

It would be very helpful if a well-designed preoperative defining system of pancreatic cancer could: (1) estimate the clinical stage of cancer (tumor extension); (2) give practical information for designing the extent of surgery and choosing the treatment modality; (3) play a role as an effective communication tool among multidisciplinary team members; and (4) help predict oncologic outcomes.

In this study, we propose a preoperative defining system in patients with pancreatic head cancer considering curative resection. Our system may be useful in improving communication and developing strategies for treating pancreatic cancer.

MATERIALS AND METHODS

Principles of the new preoperative defining system

Based on radiological interpretation of preoperative images, resected pancreatic head cancers are intended to be described as the following structures: Pancreatic head cancer; "Resectability (tumor size, cm) - Tumor location-vascular relationship (length of involved segment (cm)/Circumferential involvement (%)) - Adjacent organ involvement - Preoperative CA19-9 (initial bilirubin level) - Vascular anomaly".

Radiological resectability follows the National Comprehensive Cancer Network (NCCN) guideline^[5]. If there is no vascular involvement, no adjacent organ invasion, and no clinical metastasis, no descriptions were added to this defining system for explaining this negative information. Important abbreviations are listed in Table 1.

Organs such as the duodenum and bile duct that will be removed by standard pancreaticoduodenectomy were not described even in cases of cancer invasion. However, if the tumor invaded the pylorus or antrum, it was added as a factor of adjacent organ invasion. Figure 1 shows the examples for application of the new defining system in patients with pancreatic head cancer.

Patient data collection

From January 2008 to December 2012, medical records and preoperative image studies of patients with resected pancreatic cancer were retrospectively reviewed. The clinic-pathological variables were checked. In particular, preoperative clinical information such as resectability, radiologic tumor size, tumor location, preoperative serum CA19-9, preoperative serum bilirubin, and adjusted CA19-9 (initial serum level of CA19-9 divided by initial serum level of bilirubin)^[6] were also evaluated. The new defining system was applied to check if it could describe the tumor extent at the initial diagnosis.

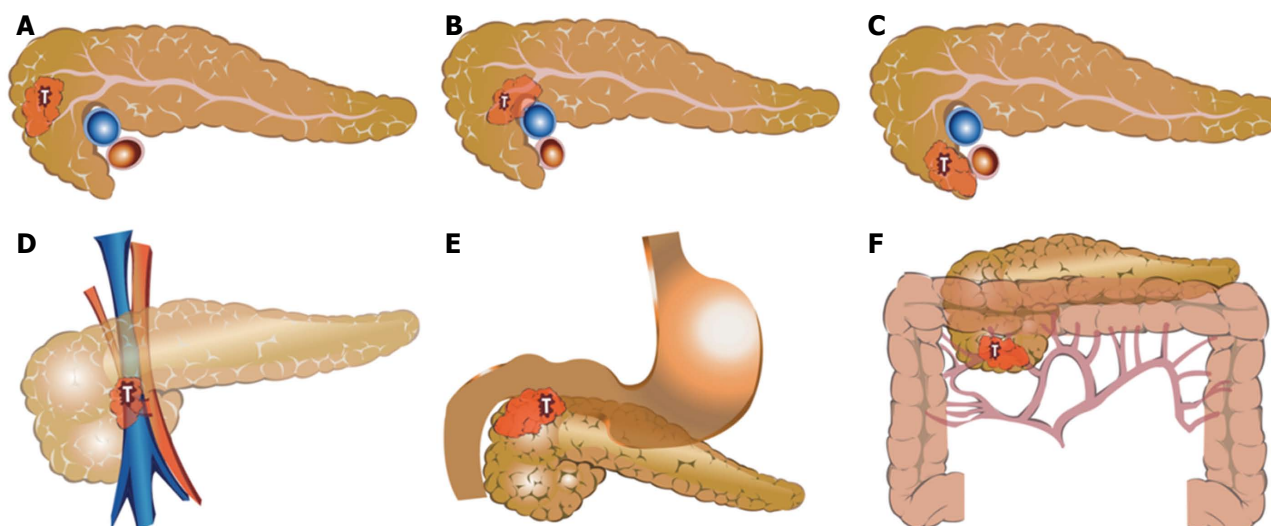


Figure 1 Clinical application of the new defining system in resected pancreatic head cancer. For example, a patient who has a 2 cm-sized tumor with preoperative CA19-9 120 U/mL and initial total bilirubin 1.2 mg/dL can be described according to the proposed new defining system as follows: (A) R2cm-H-120 (1.2); (B) BR2cm-Hn-SMV (0.5cm/30%)-120 (1.2); (C) BR2cm-Hu-SMA (0.5 cm/20%)-120 (1.2); (D) BR2cm-Hn-SMV (0.5cm/50%)-120 (1.2)-Arha; (E) R2cm-H-pylorus-120 (1.2); (F) R2cm-H-T colon mesentery-120 (1.2).

Table 1 Important abbreviations used in the new preoperative defining system for pancreatic cancer

| Symbol | Description | Comments |
|---------------------------|---|--|
| Resectability | | |
| R | Resectable | |
| BR | Borderline resectable | |
| LA | Locally advanced | |
| Location | | |
| H | Pancreatic head | Hu: uncinate process, Hn: neck portion |
| Vascular structure | | |
| CA | Celiac axis | |
| CHA | Common hepatic artery | |
| GDA | Gastroduodenal artery | OGA: origin of gastroduodenal artery |
| aRHA | Aberrant right hepatic artery | |
| Rt/Lt | Right/left | |
| SMA | Superior mesenteric artery | |
| SMV | Superior mesenteric vein | |
| SMV-SV-PV | Superior mesenteric vein-splenic vein-portal vein | |

R: Resectable pancreatic cancer; BR: Resectable pancreatic cancer; LA: Locally advanced pancreatic cancer.

Correlation between new preoperative defining system and clinico-oncologic outcomes

Individual clinical components existing in the new preoperative defining system, such as radiological resectability, tumor size, tumor location, vascular involvement, adjacent organ invasion, preoperative initial serum CA19-9 level, and initial serum bilirubin were correlated with treatment strategy and oncologic outcomes.

Statistical analysis

All statistical analyses were performed using IBM® SPSS Statistics version 20. Continuous variables were indicated as mean \pm SD and categorical variables as frequency and percentage (%). Student's *t*-tests and χ^2 tests were used. Logistic regression analysis was applied for multivariate analysis. Kaplan-Meier and Cox-proportional hazard models were applied for disease-free survival as univariate and multivariate analysis. *P* values < 0.05 were considered as statistically significant.

RESULTS

Clinical feasibility of the new preoperative defining system

During the study period, 119 patients underwent potentially curative resection of pancreatic head cancer. All were confirmed as ductal adenocarcinoma by pathologic examination. Among them, six patients without available preoperative image studies were excluded, totally 113 patients were enrolled. The new preoperative defining system was applied to describe the extent of the tumor and some clinical information for all patients. Resectable pancreatic cancer (R) was noted in 75 patients (66.4%), borderline resectable pancreatic cancer (BR) in 34 (30.1%), and locally advanced pancreatic cancer (LA) in four patients (3.5%). The mean radiologic tumor size was measured as 2.4 ± 0.8 cm in the maximum diameter. Seventy-three tumors (64.6%) were located in the pancreatic head, 35 (31%) in the uncinate process, and five (4.4%) in the pancreatic head and neck area. Forty patients (35.4%) were found to have tumors involving

Table 2 Univariate analysis to predict combined venous vascular resection in treating pancreatic head cancer

| | Combined venous vascular resection | | <i>P</i> value |
|-----------------------------------|------------------------------------|----------------|----------------|
| | No | Yes | |
| Resectability | | | |
| R | 54 | 21 | |
| BR | 17 | 17 | |
| LA | 2 | 2 | 0.054 |
| Radiologic tumor size (cm) | 2.3 ± 0.7 | 2.6 ± 0.8 | 0.044 |
| Tumor location | | | |
| Head | 49 | 24 | |
| Uncinate | 22 | 13 | |
| Including neck | 2 | 3 | 0.485 |
| Radiologic vascular component | | | |
| No | 52 | 21 | |
| Yes | 21 | 19 | 0.002 |
| Initial preoperative serum CA19-9 | 846.7 ± 2193.9 | 805.3 ± 1761.9 | 0.919 |
| Initial serum total bilirubin | 4.3 ± 5.0 | 4.9 ± 4.6 | 0.528 |

R: Resectable pancreatic cancer; BR: Resectable pancreatic cancer; LA: Locally advanced pancreatic cancer.

major vascular structures. Mean initial serum level of CA19-9 was found to be 825.7 ± 2037.8 (U/mL), and initial serum bilirubin was 4.6 ± 5.0 (mg/dL). Adjusted CA19-9 was calculated as 401.9 ± 872.8 (U/mL).

Correlation between clinical components and surgical strategy

It was found that the new preoperative defining system can help in decision-making about treatment strategies and surgical extent in pancreatic cancer management.

Thirty-nine patients (34.5%) underwent combined venous vascular resection. SMV/PV wedge resection was performed in 15 patients, and 24 patients underwent segmental resection of the PV system. Among the clinical factors used in the new preoperative defining system, radiologic tumor size, vascular components were associated with combined venous vascular resection ($P < 0.05$, Table 2). However, in multivariate analysis, only radiologic tumor size ≥ 2.4 cm [Exp(B) = 2.288, 95%CI: 1.029-5.087, $P = 0.042$] was noted to be independent clinical factor to predict combined venous vascular resection.

It was also found that resectability, radiologic tumor size, tumor location, and radiologic vascular component were related to neoadjuvant treatment before surgical resection ($P < 0.05$, Table 3). In multivariate analysis, radiologic tumor size ≥ 2.4 cm [Exp(B) = 3.608, 95%CI: 1.512-8.609, $P = 0.004$], and radiologic vascular component [Exp(B) = 5.553, 95%CI: 2.269-14.589, $P < 0.001$] were found to be independent predictive factors for preoperative neoadjuvant treatment in this study population.

Table 3 Univariate analysis to predict neoadjuvant treatment for pancreatic head cancer

| | Neoadjuvant treatment | | <i>P</i> value |
|-----------------------------------|-----------------------|-----------------|----------------|
| | No | Yes | |
| Resectability | | | |
| R | 52 | 23 | |
| BR | 10 | 24 | |
| LA | 1 | 3 | < 0.001 |
| Radiologic tumor size (cm) | 2.1 ± 5.7 | 2.7 ± 0.9 | < 0.001 |
| Tumor location | | | |
| Head | 46 | 27 | |
| Uncinate | 15 | 20 | |
| Including neck | 2 | 3 | 0.049 |
| Radiologic vascular component | | | |
| No | 52 | 21 | |
| Yes | 11 | 29 | < 0.001 |
| Initial preoperative serum CA19-9 | 600.8 ± 1640.6 | 1109.0 ± 2437.1 | 0.189 |
| Initial serum total bilirubin | 3.9 ± 4.9 | 5.5 ± 4.9 | 0.087 |

R: Resectable pancreatic cancer; BR: Resectable pancreatic cancer; LA: Locally advanced pancreatic cancer.

Correlation between clinical components and long-term oncologic outcomes

It was also noted that the proposed new defining system can be useful in predicting oncologic outcome even before confirming pathologic characteristics of the resected pancreatic cancer.

Mean disease-free survival was 24.8 mo (95%CI: 19.6-30.1) with a 5-year disease-free survival rate of 13.5%. Interestingly, when putting clinical variables used in the preoperative defining system into a Cox hazard regression model, it was found that anatomic resectability, especially borderline resectable pancreatic cancer [Exp(B) = 0.222]; radiologic tumor size ≥ 2.4 cm [Exp(B) = 1.696], tumor location, especially pancreatic head cancer involving the pancreatic neck portion [Exp(B) = 9.461]; radiologic venous vascular component [Exp(B) = 2.788]; arterial component [Exp(B) = 6.208]; initial total bilirubin ≥ 4.6 [Exp(B) = 0.588]; and adjusted CA19-9 ≥ 50 [Exp(B) = 1.972] were identified as prognostic clinical factors to predict tumor recurrence (Table 4).

DISCUSSION

TNM staging system is widely accepted, and it is aimed at predicting survival. Some kinds of cancer cannot be simplified down to a TNM stage because of unique anatomical characteristics. One of these is hilar cholangiocarcinoma (HCCA) and another is pancreas head cancer. For hilar cholangiocarcinoma, there is already a presurgical staging system that considers surrounding anatomical structures^[7]. The Jarnagin-Blumgart (J-B) classification has been used for deciding treatment plans and developing a prognosis

Table 4 Oncologic impact of clinical variables used in the new preoperative defining system

| Clinical variables | Exp(B) | 95%CI | P value |
|---------------------------------------|--------|--------------|---------|
| Resectability | | | 0.019 |
| BR | 0.222 | 0.073-0.676 | 0.008 |
| LA | 0.557 | 0.105-2.955 | 0.492 |
| Radiologic tumor size (cm) ≥ 2.4 | 1.696 | 0.993-2.897 | 0.053 |
| Tumor location | | | 0.007 |
| Hu | 0.952 | 0.557-1.629 | 0.858 |
| Hn | 9.461 | 2.634-33.976 | 0.001 |
| Vascular relationship with | | | 0.064 |
| Venous system | 2.788 | 0.952-8.165 | 0.061 |
| Arterial system | 6.208 | 1.562-24.669 | 0.010 |
| Both | 2.200 | 0.484-10.006 | 0.307 |
| CA19-9 ≥ 825 | 1.709 | 0.777-3.761 | 0.183 |
| Total bilirubin ≥ 4.6 | 0.588 | 0.339-1.022 | 0.060 |
| Adjusted CA19-9 ≥ 50 | 1.972 | 1.118-3.480 | 0.019 |

BR: Resectable pancreatic cancer; LA: Locally advanced pancreatic cancer.

of HCCA^[8]. We need a more appropriate defining system for pancreas head cancer rather than the TNM stage, something similar to the J-B classification for HCCA.

The presented new preoperative defining system can suggest the treatment strategy, extent of surgery, and even tumor biology in resected pancreatic head cancer. It was found that all resected pancreatic head cancers could be described according to the new preoperative defining system based on a preoperative CT scan. In addition, in multivariate analysis, radiologic tumor size ≥ 2.4 cm [Exp(B) = 3.608, $P = 0.004$], and radiologic vascular component [Exp(B) = 5.553, $P < 0.001$] were independent predictive factors for preoperative neoadjuvant treatment. In particular, larger tumor size (tumor size ≥ 2.4 cm) was associated with combined venous vascular resection [Exp(B) = 2.288, $P = 0.042$], suggesting that clinical components used for the currently proposed new defining system can provide important clinical clues about treatment strategy and the extent of surgery in treating pancreatic cancer.

Most importantly, the current system can predict patients' outcomes without requiring confirmation of the clinical stage of the cancer. Considering that most prognostic factors are based on pathologic characteristics^[9-13], such as lymph node metastasis, lymph node ratio, perineural invasion, lymphovascular invasion, and cell differentiation, the proposing preoperative defining system showed that even clinical characteristics, such as anatomic resectability ($P = 0.019$), tumor location ($P = 0.007$), and adjusted CA19-9 ($P = 0.019$), which can be estimated before surgical intervention, were identified as good prognostic markers for predicting tumor recurrence (Table 4).

Adjusted CA19-9 is defined as the value of initial CA19-9 level divided by serum total bilirubin. This concept was developed because the actual serum level

of CA19-9 is not reliable in patients with jaundice. We already demonstrated that adjusted CA19-9 was a prognostic clinical marker in resected pancreatic cancer^[6], which was shown again in the present study. Further clinical investigation based on a large population is necessary to define the oncologic significance of preoperative adjusted CA19-9.

When applying this new system in cases of neoadjuvant treatment, it would be easy and more subjective to detect the radiologic responsiveness after neoadjuvant treatment in borderline resectable pancreatic cancer. If a radiologist described the radiologic changes according to the new defining system, the surgeon could be well aware of the current tumor status compared with the pre-neoadjuvant treatment status, which is one of many advantages of new system. For example, it can be described in this way: BR2cm-Hu-SMV2.5cm/30%-219 (8) \rightarrow Neo-BR2cm-Hu-SMV2.0cm/10%-58 (2).

In spite of oncologic significance of lymph node metastasis, clinical N-stage (cN-stage) was not considered in this system because the accuracy of radiologic estimation of lymph node metastasis is not high^[14,15]. In addition, preoperative cholangitis, pancreatitis, and interventional approaches due to obstructive cholangio-pancreatopathy can induce secondary lymph node enlargement. In fact, lymph node metastasis is one of the important prognostic factors in resected pancreatic cancer; however, several important randomized controlled studies have proven that the extent of lymph node dissection could not contribute to increasing oncologic outcome^[16-18]. Therefore, cN-stage will not influence either prognosis or the clinical treatment strategy when the tumor is regarded as a resectable pancreatic cancer.

Instead, clinical information on the possibility of pylorus involvement or right colonic mesentery would be more useful in designing surgical extent. Recently, techniques for pyloric-ring resected pancreaticoduodenectomy^[19], subtotal stomach-preserving pancreaticoduodenectomy^[20], and combined resection of ascending colon are clinically available. In addition, descriptions of associated vascular anomaly, especially an aberrant right hepatic artery, will be another good guide for performing safe pancreaticoduodenectomy, because this artery is at risk for accidental injury during dissection of the hepatoduodenal ligament^[21]. To design an optimal operation, it is mandatory to have exact anatomical delineation preoperatively. This proposed preoperative defining system can give compact and critical anatomical information to the surgical team.

There are several important flaws in our study. First, the new defining system is only based on retrospective data of operated patients. Therefore, this could not be reflective of all patients seen for consideration of surgery. It is needed to validate this defining system with all pancreas head cancer

surgery candidates prospectively. Second, it seems to be complicated and difficult to describe. Third, this system cannot estimate actual lymph node status. However, in the era of the multidisciplinary team approach for treating pancreatic cancer, this defining system can be useful for improving communication among team members, planning the extent of surgery, developing the treatment strategy, and defining tumor biology. This system needs to be validated on different sets of patient data to confirm its clinical feasibility, reproducibility, and oncologic meanings.

COMMENTS

Background

In this multidisciplinary treatment era, it is most important to share the exact disease status among multidisciplinary team members for making appropriate treatment pathway. When it comes to pancreas head cancer, the anatomical complexity surrounding tumor can make it difficult not only to communicate with each members of team, but also to decide treatment plan. In this study, the authors suggested a preoperative defining system which contained the important anatomical and laboratory findings associated with pancreas head cancer. Then they evaluated the efficacy of this system for designing treatment plan and predict oncologic outcomes.

Research frontiers

The National Comprehensive Cancer Network (NCCN) categorized the pancreas head cancer cases into resectable, borderline resectable or unresectable diseases. But this classification solely depends on vascular relationship in the preoperative radiologic evaluation. Several studies reported that tumor characteristics, adjusted preoperative CA19-9, vascular anomalies also should be considered preoperatively.

Innovations and breakthroughs

With the suggested defining system, they authors can estimate necessary of the neoadjuvant therapy or the combined vascular resection for pancreas head cancer. Moreover, the contents of this system are strongly related to the tumor recurrence.

Applications

This study demonstrates the new defining system for pancreas head cancer and will help the multidisciplinary board to communicate with each other about the individual disease status in a comprehensive way.

Terminology

Borderline resectable pancreas cancer means there is a possibility of incomplete resection because of adjacent vital vessel invasion such as superior mesenteric vein, gastroduodenal artery, hepatic artery and superior mesenteric artery.

Peer-review

The authors recommended a good preoperative description system for pancreatic cancer patients. This proposal with possible implication in neoadjuvant treatment is very remarkable.

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Observational Study

High circulating tumor cell concentrations in a specific subtype of gastric cancer with diffuse bone metastasis at diagnosis

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Author contributions: Shibata H designed the study and wrote this manuscript; Shimazu K acquired and analyzed the data; Fukuda K, Yoshida T and Inoue M participated in acquisition and interpretation of the data.

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Informed consent statement: Informed consent and an agreement to publish were obtained from all patients.

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Data sharing statement: Participants gave informed consent for data sharing, and further the presented data are anonymized.

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Abstract

AIM: To clarify the biological feature contributing to gastric cancer with diffuse bone metastases at diagnosis.

METHODS: The participants visited the Department of Clinical Oncology, Akita University Hospital, from January 2014 to August 2015. The selection criterion for gastric cancer with diffuse bone metastases at diagnosis includes over 29 hot spots of bone scintigraphy. Circulating tumor cell were collected from 20 mL of peripheral venous blood drawn using a CellSearch kit and a CellTracks AutoPrep system by SRL, a clinical laboratory. The endpoints of this study were correlations between circulating tumor cells (CTC) count and therapeutic outcomes.

RESULTS: Among 39 patients with gastric cancer, 5 patients met the criterion. The incidence of this subtype was 12.8%. CTC counts ranged from 235 to 6440 cells/7.5 mL of peripheral blood (median of 1724). These values were much higher than common gastric cancers (2 cells). In chemo-sensitive cases, CTC counts decreased within 14 d (median) from 275, 235 and 1724 to 2, 7 and 66, respectively. On the other hand, CTC counts increased after treatment failure or insensitive case from 2, 7 and 6440 to 787, 513 and 7885, respectively. The correlation between CTC count

and survival time showed a trend, but did not reach significance ($Y = 234.6 - 0.03X$, $P = 0.085$).

CONCLUSION: High CTC count is a biological hallmark of this subtype, and can be used as a direct and definitive indicator of therapeutic outcome.

Key words: Bone metastasis; Circulating tumor cell; Gastric cancer; Predictive biomarker; Prognostic biomarker

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Core tip: It has been reported in many times that a specific subtype of gastric cancer characterized with diffuse bone metastases at diagnosis, rapid progression and poorer prognosis apparently exists in almost one of ten gastric cancers. However, the basic and biological features of this subtype are not specified until today. In this study, we identified high number of circulating tumor cell of this subtype, and considered that circulating tumor cells (CTC) is responsible for the clinical features described above. CTC is not only a biological hallmark of this subtype, but also informative as a predictive or prognostic biomarkers.

Shimazu K, Fukuda K, Yoshida T, Inoue M, Shibata H. High circulating tumor cell concentrations in a specific subtype of gastric cancer with diffuse bone metastasis at diagnosis. *World J Gastroenterol* 2016; 22(26): 6083-6088 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i26/6083.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i26.6083>

INTRODUCTION

Gastric cancer with associated diffuse bone metastases at diagnosis has rarely been reported^[1]. This condition has been referred to in the literature as "diffuse bone metastasis with hematologic disorders from gastric cancer" or "gastric cancer, initially presenting as disseminated intravascular coagulation (DIC)"^[2,3]. This condition is frequently accompanied by DIC, and the comorbidity rate with bone metastases is 82%-86%^[2,3]. Similarly, the frequency of bone metastases and gastric cancer with DIC is reported to be 87%^[1]. Rhee reported 21 patients with DIC at diagnosis among 1216 advanced gastric cancer patients, in whom 18 patients had bone metastases simultaneously ($18/1216 = 1.5\%$)^[3]. Although they are rare, they have outstanding features besides diffuse bone metastases and DIC. Additional clinical features of this subtype that differ from typical gastric cancers include a lower incidence of visceral metastases, more aggressive disease course, and poorer prognosis. For examples, Etoh reported 15 cases over the course of 20 years^[1] and Toyoshima described 5 of the 42 reported cases (11.9%)^[4]. Among the latter cases, who all had bone marrow metastases, the CTC counts

ranged from 30 to 18015 cells/7.5 mL. The reported median survival time (MST) of this disease ranges from 8 to 22 wk^[1,3,5], which is much shorter than that of more common stage IV gastric cancers, for which MSTs of 11.1 and 13.8 mo have been reported in patients treated with cisplatin plus 5-fluorouracil (5-FU) or capecitabine and trastuzumab, respectively^[6]. Accordingly, medical oncologists consider this disease to be a distinct gastric cancer subtype^[1-3], and we should know the clinicopathological characters of this subtype. The high frequency of bone metastasis with this specific subtype is in contrast to the less than 10% frequency with more common gastric cancers^[7,8]. Bone metastasis may occur once cancer cells have infiltrated the vasculature and entered the blood stream. Such circulating tumor cells (CTCs) are often detected in the peripheral blood of patients with lung, breast, and prostate cancer, which are diseases with much higher incidences of bone metastases ($36\%-73\%$)^[9]. High CTC counts have been reported for prostate and breast cancers (84 ± 885 and 75 ± 333 cells/7.5 mL in the peripheral blood, respectively)^[10]. There are also reports of a CTC count > 50 cells/7.5 mL in 14% and 10% of prostate and breast cancer patients, respectively^[10]. However, the median CTC count for patients with common forms of gastric cancer are much lower, at around 2 cells/7.5 mL, with a CTC count of > 50 cells/7.5 mL only seen in approximately 8% of gastric cancer cases^[10-12]. The characteristics of diffuse bone metastases within this subtype might reflect an increased number of CTCs. Therefore, we examined the CTC count of this specific subtype. We have previously reported CTC counts for two patients with this gastric cancer subtype^[13]. In this study, we report an additional three cases with this subtype and substantiate the clinical importance of CTCs.

MATERIALS AND METHODS

Study population and data collection

The patients, who visited and were diagnosed as gastric cancer at the Department of Clinical Oncology, Akita University Hospital, from January 2014 to August 2015, were analyzed. The selection criterion for identifying patients with this cancer subtype are as follows: (1) histopathologically confirmed gastric cancer; (2) apparent symptoms of diffuse bone metastases at onset; (3) diffuse bone metastases detected by bone scintigraphy (BS); and (4) a hot spot number over 29; This is derived from the data that the reported mean \pm SD of BS hot spot number for gastric cancer was 16 ± 13 ^[14] (Figure 1). This study was approved by the Akita University School of Medicine Ethics Committee (#828). Informed consent and an agreement to publish were obtained from all patients.

CTC collection

CTCs were isolated as previously described^[15]. In brief, CTCs were isolated from 20 mL of peripheral venous

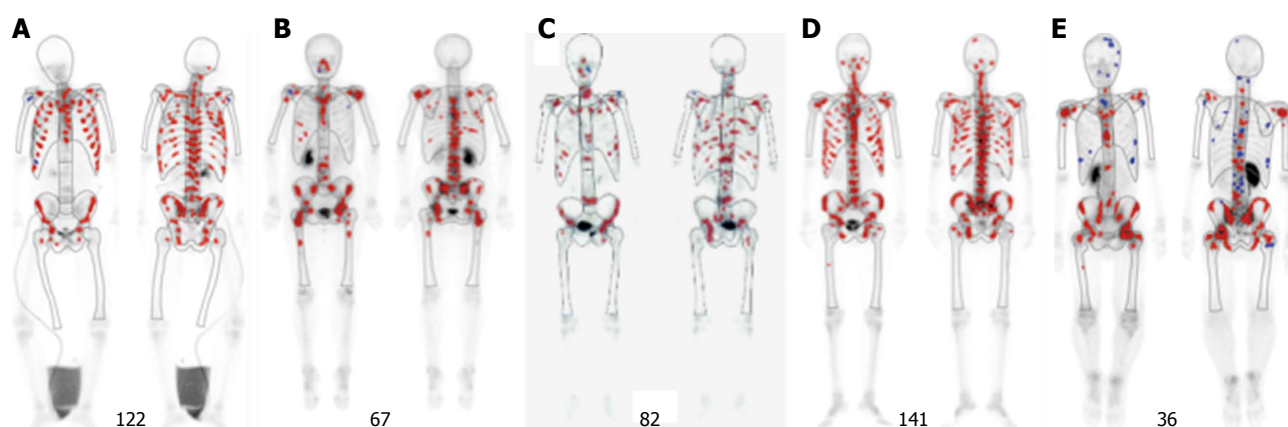


Figure 1 Diffuse bone metastases with specific gastric cancer subtypes. The results of bone scintigraphy is presented for each case (1-5 corresponds to A-E). A red dot indicates a hot spot, with the number listed at the bottom. The mean \pm SD number of hot spots was 89.6 ± 42.2 .

Table 1 Participant characteristics

| Case | Gender | Age | Hist | DIC | Distant LN metastasis | Visceral metastasis | Treatment |
|------|--------|-----|-----------------|-----|-----------------------|---------------------|------------------------|
| 1 | M | 50 | por + sig | + | - | PC | S1 + DTX ¹ |
| 2 | F | 59 | por | - | Para Aorta | LC (lung) | S1 + DTX |
| 3 | M | 78 | tub + por + sig | + | Para Aorta | - | wPTX ² |
| 4 | M | 38 | por | - | Para Aorta | - | S1 + CDDP ³ |
| 5 | M | 24 | por + sig | - | Para Aorta | PC | S1 |

¹Administrated with S1 (40 mg/m², twice daily for 14 d) and DTX (33 - 40 mg/m²) every 3 wk; ²Administrated weekly with DTX (15 mg/m²) for 3 wk; ³Administrated with S1 (40 mg/m², twice daily for 21 d) and CDDP (60 mg/m²) every 5 wk. LN: Lymph node; PC: Peritonitis carcinomatosa; LC: Lymphangitis carcinomatosa; Por: Poorly differentiated adenocarcinoma; sig: Signet ring cell carcinoma; tub: Tubular adenocarcinoma; DTX: Docetaxel; CDDP: Cis-platinum.

blood drawn using a CellSearch kit and a CellTracks AutoPrep system (Janssen Diagnostics, LLC, New Jersey, United States). This procedure was outsourced to SRL, a clinical laboratory (Tokyo, Japan).

Study endpoints

The primary endpoint of this study was CTC count and its change after chemotherapy. The secondary endpoints were correlations between CTC number and the therapeutic response, and between CTC number and survival. Evaluation of the therapeutic response was performed using response evaluation criteria in solid tumors (RECIST, version 1.1).

Statistical analysis

The values are shown as mean \pm SD. Simple regression analysis was performed using StatMate III, version 3.14 (ATMS, Tokyo, Japan). This statistical method was reviewed by Professor Katsuyuki Murata from Department of Environmental Health Sciences, Graduate School of Medicine, Akita University.

RESULTS

Characteristics of a specific subtype of gastric cancer with diffuse bone metastasis at diagnosis

During this period, 39 patients with gastric cancer (28 males and 11 females) visited our department.

Five patients met the criterion, and were diagnosed as this subtype. The incidence of this subtype was 12.8%. They included four males and one female, who aged 24-78 years (median, 50 years) (Table 1). Patients were histopathologically diagnosed with adenocarcinomas, signet ring cell carcinomas, or mixed cancers. Distant metastases other than bone metastases are reported in Table 1. DIC was observed in two cases.

CTC counts of this subtype

CTC counts before chemotherapy ranged from 235 to 6440 cells/7.5 mL of peripheral blood (median of 1724; Table 2), which is considered to be a characteristic of this gastric cancer subtype. These values were considerably higher than is typically found with more common gastric cancers, which have a reported median value of 2 cells/7.5 mL^[11].

Change of CTC count and therapeutic response

The therapeutic course for each case is presented in Table 2. Cases 1 and 2 were described in detail elsewhere^[13]. With three cases (cases 1, 2, and 4), tumors were sensitive to chemotherapy administered immediately after the CTC examination. Tumor response was evaluated by computed tomography (CT) imaging performed nearly 3 mo after the initiation of chemotherapy. The patient CTC count was reassessed

Table 2 Circulating tumor cells count and therapeutic outcomes

| Case | Tumor markers | | CTC count | | Date ¹ (the X d) | Effects | Survival (d) ² |
|------|---------------------------|---------------|---------------------------|--|-----------------------------|---------------|---------------------------|
| | pre (-) post ³ | | pre (-) post ³ | | | | |
| 1 | CEA | 288 (↓) 160 | 275 (↓) 2 | | 16 | non CR/non PD | 160 |
| | CA19-9 | 158 (↑) 690 | | | | | |
| 2 | CEA | 120 (↓) 83 | 235 (↓) 7 | | 11 | (+) PR | 246 |
| | CA19-9 | 5201 (↑) 6543 | | | | | |
| 3 | CEA | 1.4 (→) 1.3 | 6440 (↑) 7885 | | 14 | (-) PD | 30 |
| | CA19-9 | 7.6 (→) 8.3 | | | | | |
| 4 | CEA | 15 (↓) 1.1 | 1724 (↓) 66 | | 14 | (+) PR | Alive > 120 |
| | CA19-9 | 4.2 (→) 4.1 | | | | | |
| 5 | CEA | 2.4 (↑) 12 | 4197 -ND | | ND | (+) PD | 31 |
| | CA19-9 | 205 (↑) 653 | | | | | |

¹The days of evaluation of tumor markers and CTC from the start of treatment; ²The days from the start of the treatment to death. CTC count is indicated as cells/7.5 mL. The units of CEA and CA19-9 are ng/mL and U/mL, respectively; ³The previous and post treatment values are indicated as pre - post. ↓: Decrease; ↑: Increase; →: No change. ND: Not done; Non CR/non PD: Case 1 has no target lesions; PR: Partial response; PD: Progressive disease.

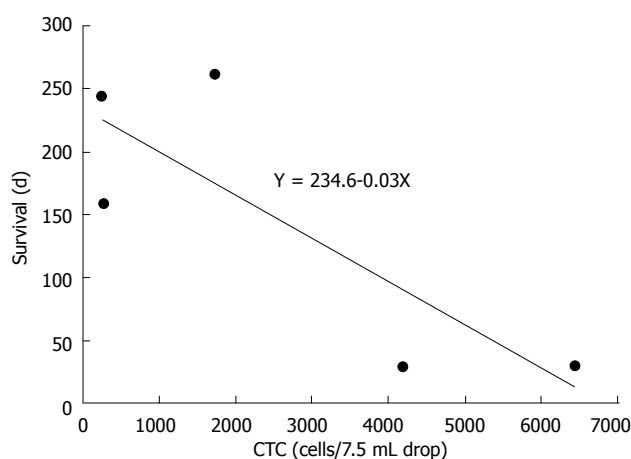


Figure 2 Correlation between the circulating tumor cells count and survival time. Survival time likely correlates with the initial circulating tumor cells (CTC) count ($P = 0.085$).

at a median of 14 d after chemotherapy (range, 11-16 d). A change in the CTC count may be an earlier indicator of the therapeutic outcome than changes visible upon imaging. Earlier detection could be critical because the progression of this gastric cancer subtype tends to be very rapid. If a tumor is insensitive to treatment, an alternative chemotherapeutic agent may be substituted after only one cycle, eliminating the need to continue an ineffective systemically administered treatment for several months, as is necessary to detect imaging changes. Moreover, this cancer subtype often lacks measurable targets except lymph node metastases, making imaging evaluation difficult. Furthermore, concurrent measurement of the serum tumor markers carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) produced considerably different results in two cases (cases 1 and 2; Table 2). For these cases, it was not possible to predict the therapeutic responses based on changes in these markers. Nevertheless, the CTC count can be used as a direct and definitive indicator

of therapeutic outcome in this gastric cancer subtype. Alternatively, CTC counts were increased in cases 1 and 2 upon treatment failure. For those cases, CTC counts increased to 787 and 513 cells/7.5 mL peripheral blood, respectively. An additional patient (case 3) who was insensitive to the initial treatment showed an increase in the CTC count from 6440 to 7885 cells/7.5 mL peripheral blood. A second course of chemotherapy was not initiated because of a worsened general condition.

Correlation between CTC count and survival

The peripheral blood CTC count also can predict patient survival. For case 5, chemotherapy was only administered for 3 d because of the patient's rapidly worsening condition. With the exception of case 4 (still alive for > 180 d), the survival times for the other 4 cases appeared to correlate with their initial CTC count (Figure 2). The initial CTC counts were high for the two short-term survivors (cases 3 and 5) who lived until 30 d after their initial diagnosis (6440 and 4197 cells/7.5 mL peripheral blood, respectively). The long-term survivors who lived for more than 160 d (cases 1, 2, and 4) had considerably lower initial CTC counts (235, 275, and 1724 cells/7.5 mL peripheral blood, respectively). Although case 4 is still alive (over 263 d), the relationship between CTC count and survival time showed a negative trend but did not reach significance ($Y = 234.6 - 0.03X$, $P = 0.085$; Figure 2). Concerning case 4, the CTC was additionally examined 2 times during this period. Those are suppressed, and they were 33 and 60 cells/7.5 mL, respectively. That indicates the first line chemotherapy S1 plus cisplatin is still effective for case 4. These observations suggest that the initial CTC count is a useful prognostic biomarker for patients with this disease.

DISCUSSION

Our results indicate that, unlike typical gastric cancer,

the subtype of gastric cancer that presents with diffuse bone metastases can be characterized as having a high CTC count. Therefore, we propose this subtype of gastric cancer as circulating gastric cancer (cGC). The incidence of cGC is roughly estimated to range from 1.5% to 11.9% of gastric cancer in the literature^[3,4]. However, if CTC count is characteristics of this subtype, we can estimate the incidence more precisely. One of the reasons why cGC metastasizes to bone with high frequency is due to the blood stream from stomach, we considered. The blood stream from almost digestive tracts other than upper stomach and lower rectum flows into portal vein. However, a part of the blood stream from the proximal region of stomach flows into connecting vein between left gastric vein and esophageal vein, and leads to valveless Batson venous plexus, which forms venous plexus penetrating spines *via* azygous and hemiazygous veins^[16]. This situation is similar to the blood stream from prostate, breast and lung. These high CTC counts may have contributed to the diffuse bone metastases observed with this subtype. Bone marrow is considered to be a common and easily accessed homing organ for tumor cells that escape epithelial tumors^[17]. As the other factor for CTC to metastasize to bone, it is thought there are niches in the bone marrow where CTCs can easily reside, and the bone marrow is considered to be a reservoir for disseminated tumor cells^[4,18]. It has also been suggested that cancer cells metastasize to the bone through a multistep process^[18]. Ongoing research efforts may define the molecular basis of this subtype in the near future. There may be specific genetic mutations present in CTCs that confer the specific phenotypes that enable bone metastasis. In addition, these mutations may represent the characteristic features of this subtype that facilitate the rapid progression of circulating cells to the bone. Thus, CTC analysis might establish the molecular pathogenesis of this specific cancer subtype. Within this subtype, in all practicality, the CTC count may serve as a definitive biomarker for evaluating therapeutic effects, as has been previously suggested^[19,20]. The CTC count has the further advantage of allowing the evaluation of chemotherapeutic benefit earlier than imaging measures. Furthermore, CTC measurement may assist in predicting patient survival. In spite of rareness of the incidence of cGC as low as 10%, further and larger study should be warranted in near future.

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COMMENTS

Background

The incidence of subtype of gastric cancer that presents with diffuse bone metastases at onset is roughly estimated as 10% or less of gastric cancer.

However, the biological natures of this disease are not identified, and also this situation is not clearly defined.

Research frontiers

Recently to examine cancer patients, liquid biopsy is very accessible and becomes a reliable way in which the authors can get DNA and RNA of cancer cells or even capture themselves from blood drawn. By this easy way, the authors can get any biological information of the cancer cells' situation at real time.

Innovations and breakthroughs

Concerning this subtype of gastric cancer, no one argues the high number or importance of circulation tumor cell (CTC) for diagnosis. The authors also claim that CTC of this subtype is very useful as predictive and prognosis biomarkers.

Applications

The CTC count of this subtype should be measured prior to administration of chemotherapeutic agents. Then it should be reexamined just after one cycle of chemotherapy, and compared them to evaluate the sensitivity of the drugs used. That could result in a better outcome to the patient.

Terminology

CTC is a living cancer cell in the patient's blood flow. It can be captured by immunomagnetic beads coated suitable antibodies.

Peer-review

Shimazu *et al* described their clinical experience with 5 cases of a rare type of gastric cancer characterized by diffuse bone metastases at diagnosis, rapid progression and poor prognosis. They identified high number of CTC in this type of cancer, and considered that CTC is responsible for the clinical features. This is also an extension of their previous report on 2 cases included in the study.

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Henoch-Schönlein purpura from vasculitis to intestinal perforation: A case report and literature review

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Abstract

Henoch-Schönlein purpura (HSP) is generally a self-limited vasculitis disease and has a good prognosis. We report a 4-year-old Thai boy who presented with palpable purpura, abdominal colicky pain, seizure, and eventually developed intestinal ischemia and perforation despite adequate treatment, including corticosteroid and intravenous immunoglobulin therapy. Imaging modalities, including ultrasonography and contrast-enhanced computed tomography, could not detect intestinal ischemia prior to perforation. In this patient, we also postulated that vasculitis-induced mucosal

ischemia was a cause of the ulcer, leading to intestinal perforation, and high-dose corticosteroid could have been a contributing factor since the histopathology revealed depletion of lymphoid follicles. Intestinal perforation in HSP is rare, but life-threatening. Close monitoring and thorough clinical evaluation are essential to detect bowel ischemia before perforation, particularly in HSP patients who have hematochezia, persistent localized abdominal tenderness and guarding. In highly suspicious cases, exploratory laparotomy may be needed for the definite diagnosis and prevention of further complications.

Key words: Henoch-Schönlein purpura; Corticosteroids; Vasculitis; Intestinal perforation; Bowel ischemia; Peritonitis

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Core tip: Henoch-Schönlein purpura (HSP) with intestinal perforation during the course of corticosteroid treatment was a rare condition. We report a HSP patient with vasculitis-induced mucosal ischemia, eventually leading to ulcer and intestinal perforation. No imaging modality could detect bowel ischemia before perforation. Histopathology of resected bowel also suggested that high-dose corticosteroids might be a contributing factor to intestinal perforation in this patient. Early treatment by surgical resection of ischemic bowel can prevent intestinal perforation and peritonitis, which are life-threatening complications.

Lerkvaleekul B, Treepongkaruna S, Saisawat P, Thanachattachairattana P, Angkathunyakul N, Ruangwattanapaisarn N, Vilaiyuk S. Henoch-Schönlein purpura from vasculitis to intestinal perforation: A case report and literature review. *World J Gastroenterol* 2016; 22(26): 6089-6094 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i26/6089.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i26.6089>

INTRODUCTION

Henoch-Schönlein purpura (HSP) is the most common childhood vasculitis disorder and is characterized by palpable purpura, arthritis or arthralgia, abdominal colicky pain, and nephritis. It is multi-systemic small vessel vasculitis, 50%-80% of which typically involves the gastrointestinal tract (GI), causing diffuse colicky pain due to submucosal or subserosal hemorrhage and edema. This GI disease is generally benign and has a good response to corticosteroid therapy in most cases. However, severe complications such as intussusception, massive GI bleeding, and intestinal perforation can occur. HSP-associated intestinal perforation is a rare complication but it is life-threatening and mortalities have been reported^[1]. We present here a pediatric patient with severe HSP who developed intestinal

ischemia and perforation without intussusception despite early and adequate corticosteroid therapy.

CASE REPORT

A 4-year-old Thai boy was referred to our hospital due to persistent severe abdominal pain and seizure. He had been hospitalized at a private hospital due to colicky abdominal pain and palpable purpura for two days prior to admission. On admission, the abdomen was soft on palpation and not tender. Furthermore, there was palpable purpuric rash on both legs. His CBC revealed hemoglobin 125 g/L, WBC $11.4 \times 10^9/L$, platelet $501 \times 10^9/L$. ESR was 14 mm/h. Kidney function was normal; BUN was 1.99 mmol/L, Creatinine was 23 $\mu\text{mol/L}$, and the urinary analysis showed specific gravity 1.020, no protein, no glucose, no blood, no bilirubin, WBC 0-1 cells/HPF, RBC 0-1 cells/HPF. The serum electrolyte showed sodium 132 mmol/L, potassium 4.2 mmol/L, chloride 94 mmol/L, bicarbonate 17.2 mmol/L, and the skin biopsy demonstrated small vessel vasculitis. Therefore, HSP was diagnosed and intravenous methylprednisolone (IVMP) 2 mg/kg per day was started promptly. His abdominal pain had improved after the treatment, however two days later (D5) the pain recurred and was progressive. Abdominal ultrasonography revealed no intussusception. Abdominal computed tomography (CT) showed mild bowel wall edema at duodenum and proximal jejunum. On day six, he developed status epilepticus and alteration of consciousness. He was intubated and put on ventilator support. Antiepileptic drug treatment was started. While he was in an euvoletic state, his serum sodium was 120 mmol/L, urine osmolality 100 mmol/kg, and serum osmolality 256 mmol/kg. The etiology of seizure was thought to be hyponatremia due to syndrome of inappropriate antidiuretic hormone. MRI and MRA of the brain revealed no evidence of central nervous system (CNS) vasculitis. CSF fluid analysis was normal. Blood tests for anti-nuclear antibody, anti-double stranded DNA, and C3 were normal. Since CNS vasculitis could not be excluded, one dose of pulse methylprednisolone (MP) 30 mg/kg was commenced. Then he was referred to our hospital for further management. On the first day of presentation in our hospital (D7), his physical examination revealed drowsiness and no abdominal distension or guarding. CBC revealed thrombocytosis ($553 \times 10^9/L$), WBC $10 \times 10^9/L$, and hemoglobin 110 g/L. ESR and CRP were 32 mm/h and 5 mg/L respectively. Urinalysis and serum creatinine level were normal. He subsequently regained consciousness and was extubated after serum sodium returned to normal level. The EEG was normal, therefore antiepileptic drug treatment was discontinued. The purpura faded away and the abdominal pain markedly improved, therefore IVMP was continued at the dose of 2 mg/kg per day. Abdominal pain recurred five days after admission to our hospital (D12) with

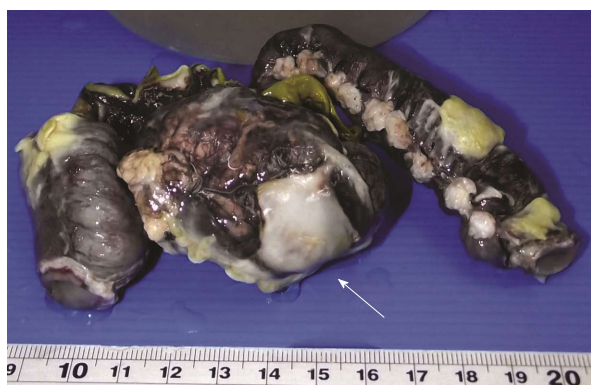


Figure 1 Specimen after fixation revealed a perforated ulcer with fibrofibrinous exudate (arrow).

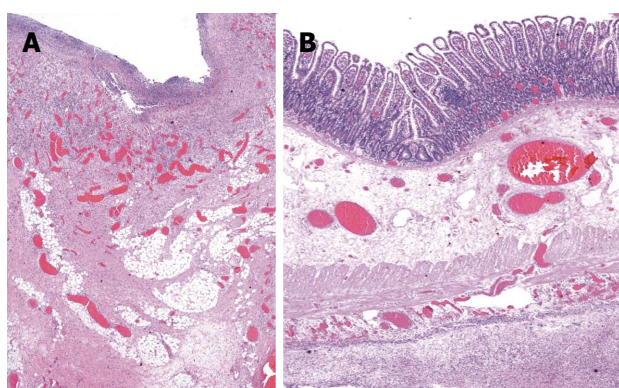


Figure 2 Histopathologic findings (hematoxylin-eosin staining, magnification $\times 40$). A: Base of ulcer revealed inflammation, granulation tissue and fibrosis; B: Remaining ileum showed lymphoid follicle depletion, submucosal and serosal congestion with diffuse peritonitis.

tenderness and voluntary guarding at the right lower quadrant. Abdominal ultrasound revealed diffuse small bowel wall thickening and minimal ascites without intussusception. Corticosteroid resistant HSP was considered, and intravenous immunoglobulin (IVIG) 2 g/kg was initiated. Two days later (D14), he developed hematochezia with tenderness and voluntary guarding at the right lower quadrant. Intestinal ischemia was suspected. A contrast-enhanced CT of the whole abdomen was performed and revealed a long segment of mild circumferential bowel wall thickening with preserved wall enhancement involving jejunum and ileum, but the evidence of bowel ischemia was inconclusive. He was also evaluated by pediatric surgeons and the continuation of medical treatment was suggested. Three days later (D17), he developed signs of peritonitis with abdominal distension, generalized guarding and tenderness. His body temperature was 38.3°C , blood pressure was 100/60 mmHg, pulse rate was 130 beats/min, and respiratory rate was 26 breaths/min. Upright abdominal radiograph demonstrated intraperitoneal free air. An emergency exploratory laparotomy was performed and revealed ischemic ileum of 35 cm at 35 cm proximal to the ileocecal valve and large perforated ulcers, size 3.5

cm \times 1.5 cm, on the ischemic bowel. The pathological examination macroscopically revealed a large perforated ulcer with fibrofibrinous exudates (Figure 1). The histopathology revealed mixed inflammatory cell infiltrate with granulation tissue and fibrosis at the base of ulcer. The remaining bowel showed depletion of lymphoid follicles (Peyer's patch), submucosal and subserosal congestion and diffuse peritonitis (Figure 2). After surgery, his general symptoms gradually improved and abdominal pain resolved. IVMP was switched to oral prednisolone, and then discontinued in week 3. The patient was discharged thirteen days after surgery (D31). He has been well without disease recurrence at the 4 mo follow-up. The summaries of clinical data, investigations, and treatment during the course of disease are shown in Table 1.

DISCUSSION

This patient developed intestinal perforation due to a bowel ischemia-related ulcer at day 17 after the onset of disease despite receiving corticosteroid and IVIG therapy. HSP-associated intestinal perforation is a rare complication with an estimated prevalence of 0.38%^[2]. To the best of our knowledge, only 11 pediatric cases have been reported in the English literature (Table 2). The most common site of perforation is the small intestine, particularly the ileum followed by the jejunum. The pathogenesis of bowel perforation may result from vasculitis-induced thrombosis, leading to ischemia and consequently total necrosis of the bowel wall^[1]. In addition, intussusception and spontaneous bowel perforation can concurrently occur in children with HSP^[3-6].

Treatment of HSP remains controversial. Patients with mild GI disease may recover without any therapy. Corticosteroids have been used in patients with moderate to severe GI involvement as the standard therapy, whereas IVIG is an alternative treatment for steroid-resistant disease^[7,8]. Other reported treatment for refractory GI diseases in HSP were plasma exchange and immunosuppressive drugs, including cyclophosphamide, azathioprine, and cyclosporine^[9]. Early corticosteroid therapy in HSP patients results in better resolution of abdominal pain within 24 h and reduces the risk of persistent renal disease^[8]. In contrast, some studies demonstrated that corticosteroids may increase the risk of bowel perforation through the reduction of mucosal renewal and lymphoid patches^[2]. A presentation of persistent severe abdominal pain despite corticosteroid therapy is uncommon and could be due to refractory disease, intussusception and intestinal ischemia. In our case, we postulated that vasculitis-induced mucosal ischemia was the cause of the ulcer which subsequently perforated, leading to diffuse peritonitis. Based on the histopathological findings of granulation tissue and fibrosis at the base of ulcer, we inferred that the perforation happened some time prior to

Table 1 Summaries of clinical data, investigations, and treatment in this case report

| Category | D1-3 8-10/1/15 Private hospital | D5 12/1/15 | D6 13/1/15 | D7 14/1/15 This hospital | D12 19/1/15 | D14 21/1/15 | D17 24/1/15 | D31 7/2/15 |
|-----------------|--|--------------------------------|--|---|---|--|--|------------------------------------|
| Symptoms | Intermittent abdominal pain, purpuric rash | Abdominal pain progressed | Seizure | Regained consciousness, abdominal pain improved, purpura faded away | Abdominal pain progressed | Persistent abdominal pain with hematochezia | Low grade fever, severe abdominal pain | No abdominal pain, normal appetite |
| Abdominal signs | Soft, mild tenderness | Generalized voluntary guarding | | Soft, not tender | Localized guarding and tenderness at RLQ | Localized guarding and tenderness at RLQ | Distend, generalized guarding | Soft, not tender |
| Investigations | | US - no intussusception | Hyponatremia MRI/MRA - no vasculitis, CSF fluid - normal | Normal EEG | US - Diffuse small bowel wall thickening, minimal ascites, no intussusception | Radiograph - no free air, CT - bowel wall thickening, normal homogeneous enhancement | Radiograph - intraperitoneal free air | |
| IVMP (mg/kg/d) | 2 | 1 | 30 | 2 | 2 | 2 | 2 | Switch to oral prednisolone |
| Treatment | | Nexium, sucralfate | ET tube, 3% NaCl, Keppra, Refer | Extubation, off Keppra | NPO, IVIG 2 gm/kg/dose | NPO | NPO, ATB, exploratory laparotomy | |

RLQ: Right lower quadrant; US: Ultrasonography; CT: Computerized tomography; EEG: Electroencephalogram; IVIG: Intravenous immunoglobulin; IVMP: Intravenous methylprednisolone; NPO: Nothing by mouth; ATB: Antibiotic; ET: Endotracheal; NaCl: Sodium chloride.

surgery. However, the exact time of perforation cannot be accurately estimated. It is likely that he had concealed bowel perforation, masking the symptoms and signs and leading to a delay in detecting the bowel perforation. We also speculated that high-dose corticosteroid treatment was a contributing factor due to reducing mucosal lymphoid follicles (Peyer's patches) and compromising mucosal renewal and healing.

Early detection of intestinal ischemia is crucial for early surgical resection. Delayed diagnosis can result in intestinal perforation and peritonitis which are severe and life-threatening complications. Unfortunately, the signs and symptoms of intestinal ischemia are not specific, ranging from decreased bowel sound, abdominal tenderness, abdominal distension, to hematochezia. Moreover, some patients had transient improvement during the course of disease^[2]. The gold standard investigation for intestinal ischemia is arteriography but it is invasive and difficult to perform in children. CT scan, particularly multidetector computed tomography, is a major investigation in the diagnosis of bowel ischemia. The diagnostic findings in CT scan include decreased or absent bowel wall enhancement and intramural gas in the bowel wall, however, these findings are found infrequently. Frequent findings are thickening of intestinal wall, dilatation of lumen, and intra-abdominal fluid, which cannot be differentiated from other diseases^[10]. Therefore, bowel ischemia cannot be excluded based on the absence of intramural gas in the bowel wall

from a contrast-enhanced CT scan, as seen in this patient. A plain abdominal radiograph is useful only in cases of perforation but it could not detect bowel ischemia. Abdominal ultrasound is more frequently used to identify other abdominal pathology, such as intussusception and bowel perforation. However, this technique is limited if the patient has a large amount of air in the bowel loops. Ultrasonography may be useful in the late phase of ischemic bowel by demonstrating bowel wall thickening, a fluid-filled lumen, decreased or absent bowel movement, and extra-luminal fluid^[11]. Since bowel perforation can occur at 1 to 4 wk after initial presentation^[1,6,9-15], close observation and frequent physical examination are essential for the early detection of bowel ischemia, especially when the patients have hematochezia, persistent localized abdominal tenderness, and guarding. Finally, in highly suspicious patients, exploratory laparotomy may be needed for the definite diagnosis and treatment of intestinal ischemia and bowel perforation.

In conclusion, HSP is generally a self-limited disease and rarely requires surgical intervention. Corticosteroid therapy is indicated in moderate to severe GI involvement. Persistent severe abdominal pain after initiating corticosteroid therapy is uncommon and physicians should be concerned about GI complications. Bowel ischemia and perforation are rare but life-threatening conditions. Therefore, early diagnosis by clinical evaluation and proper investigations, and prompt surgical treatment are

Table 2 The characteristics and outcome in pediatric patients with Henoch-Schönlein purpura associated-intestinal perforation

| Ref. | Age (yr) | Sex | Interval between the onset of symptoms and perforation | Location of the perforation | Associated findings | Treatment before perforation | Symptoms before perforation | Reason for surgery | Outcome |
|--|----------|-----|--|-----------------------------|-----------------------------------|------------------------------|--------------------------------------|------------------------------------|---------|
| Choong <i>et al</i> ^[1] | 5.0 | M | 16 d | Ileum | Intussusception (ileocolic) | Prednisolone | Fever | Peritonitis | Survive |
| Shiohama <i>et al</i> ^[6] | 7.0 | M | 15 d | Distal ileum | No | Prednisolone | Melena | Subphrenic free air | Survive |
| | 11.0 | M | 11 d | Jejunum and ileum | No | Prednisolone | Melena, hematemesis | Abdominal rigidity | Survive |
| Başaran <i>et al</i> ^[9] | 4.5 | M | > 15 d | Ileum | Intussusception (ileoileal) | NA | NA | Free air below the right diaphragm | Death |
| | 8.5 | M | > 10 d | Distal ileum | Small bowel adhesions | Prednisolone | NA | Free air in peritoneal cavity | Death |
| Blachar <i>et al</i> ^[10] | 5.0 | M | 28 d | Distal ileum | Intussusception (jejunojejunal) | Hydrocortisone | Hematochezia, hematemesis, fever | Generalized peritonitis | Survive |
| Reginelli <i>et al</i> ^[11] | 4.0 | F | 19 d | Ileum | Bowel necrosis | Cortisol hemisuccinate | Melena, fever | Peritonitis | Survive |
| Rodriguez-Erdmann <i>et al</i> ^[12] | 5.0 | M | > 12 d | Proximal ileum | Intussusception (ileocolic) | Prednisolone | Fever | Abdominal rigidity | Survive |
| Law <i>et al</i> ^[13] | 3.0 | M | 4 wk | Ileum | Ileal stricture | Prednisolone | Fever | Pneumoperitoneum | Survive |
| Yigiter <i>et al</i> ^[14] | 13.0 | M | 7 d | Ileum | Ileal necrosis | Prednisolone | Bile-stained vomiting, bloody stools | Peritonitis | Survive |
| Wang <i>et al</i> ^[15] | 5.0 | F | 22 d | Terminal ileum | Intestinal necrosis, and adhesion | Broad spectrum antibiotics | Abdominal bulge | Abdominal rigidity | Death |
| This case | 4.0 | M | 21 d | Ileum | Intestinal ischemia, necrosis | IVMP IVIG | Hematochezia, RLQ pain and guarding | Subphrenic free air | Survive |

NA: Not available; IVMP: Intravenous methylprednisolone; IVIG: Intravenous immunoglobulin; RLQ: Right lower quadrant.

necessary for decreasing morbidity and mortality in these patients.

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COMMENTS

Case characteristics

A 4-year-old Thai boy, who was diagnosed with Henoch Schönlein purpura (HSP), had persistent severe abdominal pain despite receiving corticosteroid therapy.

Clinical diagnosis

Physical examination revealed abdominal distention, localized guarding and tenderness.

Differential diagnosis

Gastrointestinal vasculitis from refractory HSP, intussusception, intestinal

ischemia.

Laboratory diagnosis

Acute phase reactants were elevated.

Imaging diagnosis

Upright abdominal radiograph demonstrated intraperitoneal free air.

Pathological diagnosis

A large perforated ulcer with fibrofibrinous exudates and the remaining bowel showed depletion of lymphoid follicles (Peyer's patch), submucosal and subserosal congestion and diffuse peritonitis.

Treatment

Exploratory laparotomy with ileal resection and end to end ileoileostomy.

Related reports

HSP with intestinal perforation is a rare condition. To the best of our knowledge, only 11 pediatric cases have been reported in the English literature. The literature reports showed that bowel perforation can occur 1 to 4 wk after initial presentation.

Term explanation

Syndrome of inappropriate antidiuretic hormone secretion is the condition that causes increased secretion or enhanced antidiuretic hormone, resulting in

excess fluid in the body and leading to hyponatremia.

Experiences and lessons

Despite adequate corticosteroid therapy and non-specific findings in the abdominal computed tomography, intestinal ischemia could not be excluded especially in HSP patients with hematochezia, persistent severe abdominal pain and localized guarding.

Peer-review

This is a very interesting case report and review of the literature. The authors did a very nice job of presenting all of the pertinent information from the case. Additionally, the discussion was well-balanced and provided needed information. The table and figures were also appropriate for the case and manuscript. Even if the manuscript doesn't present important new methods or novel findings, it reminds pediatricians and gastroenterologists that close observation and frequent physical examination are essential for the early detection of bowel ischemia as possible cause of bowel perforation.

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