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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Role of cardiovascular intervention as a bridge to liver transplantation

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Abstract

End stage liver disease (ESLD) is associated with many specific derangements in cardiovascular physiology, which influence perioperative outcomes and may profoundly influence diagnostic and management strategies in the preoperative period. This review focuses on evidence-based diagnosis and management of coronary, hemodynamic and pulmonary vascular disease in this population with an emphasis on specific strategies that may provide a bridge to transplantation. Specifically, we address the underlying prevalence of cardiovascular disease states in the ESLD population, and relevant diagnostic criteria thereof. We highlight traditional and non-traditional predictors of cardiovascular outcomes following liver transplant, as well as data to guide risk-factor based diagnostic strategies. We go on to discuss the alterations in cardiovascular physiology which influence positive- and negative-predictive values of standard noninvasive testing modalities in the ESLD population, and review the data regarding the safety and efficacy of invasive testing in the face of ESLD and its co-morbidities. Finally, based upon the totality of available data, we outline an evidence-based approach for the management of ischemia, heart failure and pulmonary vascular disease in this population. It is our hope that such evidence-driven strategies can be employed to more safely bridge appropriate candidates to liver transplant, and to improve their cardiovascular health and outcomes in the peri-operative period.

Core tip: The population of liver transplant candidates is rapidly evolving with respect to advanced age, etiology and co-morbidities. Consequently, the cardiovascular risk profiles of these candidates have increased. At the same time, the availability of interventions, both mechanical and pharmacologic, for cardiovascular conditions has allowed previously unsuitable candidates to go on to liver transplantation. Therefore, it is imperative to understand how to define the cardiovacular risk profile of liver transplant candidates and the pre-transplant treatment options available to them.

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CORONARY ARTERY DISEASE

In light of the rising prevalence of coronary artery disease (CAD) among the general population, and the

increasing age and co-morbidities among present-day liver transplant candidates, special care must be given to coronary evaluation and management prior to liver transplantation (LT)[1]. It has been estimated that more than one in four LT candidates with traditional coronary risk factors (Table 1) may have developed moderate coronary stenosis by the time of LT consideration even while asymptomatic and that the likelihood of obstructive CAD (> 70% coronary stenosis or > 50% left main stenosis) is greatest among candidates with ≥ 2 traditional cardiac risk factors[2-4]. In particular, age > 50 years and diabetes mellitus (DM) seem predictive of cardiac ischemia post-LT[5-9]. In one analysis, LT recipients with known CAD or DM had approximately 40% greater 5-year mortality than those without CAD or DM[10]. In a retrospective analysis of ESLD patients who underwent invasive angiography prior to LT, multi-vessel CAD of any severity was associated with increased mortality and postoperative hemodynamic instability[11]. It is therefore important to identify and treat patients at risk for coronary disease prior to liver transplantation given their elevated risk of postoperative ischemic complications[9,12,13].

Ischemic evaluation with exercise or pharmacologic stress testing (utilizing either echocardiographic or perfusion imaging) has been shown to have decreased predictive value in LT candidates when compared to the general population. (Table 2) Stress testing should be pursued based on careful, individualized evaluation of the candidate's pretest probability for having CAD. In general, the ability to exercise to target heart rate is blunted in LT candidates, likely due to decreased beta-agonist transduction, and pharmacologic stress testing is usually favored[14]. For the same reason, LT candidates may not achieve desired chronotropy on dobutamine stress echocardiography (DSE). Indeed, those who do not achieve target heart rate or peak double product (heart rate × blood pressure) are felt to be at elevated risk of postoperative cardiovascular events[15]. DSE may have poor sensitivity (reported as low as 13%) and low negative predictive value (reported as low as 75%) among LT candidates[16-18]. Vasodilator perfusion imaging with nuclear SPECT (single photon emission computed tomography) may also have limited utility in the setting of the chronically vasodilated states seen in advanced liver disease[19]. Resting microvascular vasodilation in ESLD may effectively "decrease" available coronary flow reserve, which may in turn lead to apparent perfusion defects having lower-than-expected specificity for obstructive epicardial coronary disease (*i.e.*, false-positive vasodilator stress test results)[20-24] (Table 2).

Non-alcoholic steatohepatitis (NASH) cirrhotic patients more commonly exhibit traditional coronary risk factors and associated CAD compared to patients with other etiologies of cirrhosis[25-29]. In addition, cirrhotic patients with concomitant renal dysfunction are also at elevated risk for coronary disease[30].

A direct visual assessment of the coronary artery anatomy should be considered for LT candidates with high pretest probability of CAD (≥ 2 traditional coronary risk factors, especially DM) in addition to those with ischemia on stress imaging. In one study of LT candidates without known CAD, greater than 30% were found to have at least moderate CAD on cardiac CT angiography[4]. CT angiography offers an attractive balance of safely and accuracy for this purpose. It can be offered to patients of normal habitus who are able to lie still, perform required breath holding maneuvers and who have a regular non-tachycardic rhythm[4,31,32]. Negative predictive value of normal or nonobstructive findings on coronary CT angiography can approach 95%[33]. Invasive coronary angiography can also be performed safely in LT candidates even in the face of renal dysfunction and elevated bleeding risk[34-36]. Invasive angiography *via* a transradial approach has been advocated in LT candidates, if possible, to promote more reliable hemostasis in the setting of the often-profound coagulopathies and cytopenias seen with ESLD[37,38]. Revascularization of obstructive coronary disease may be pursued to improve symptom burden and cardiovascular mortality per ACC/AHA guidelines, and in cases where the burden of obstructive CAD would prohibit LT in an otherwise appropriate surgical candidate[24,39,40].

Revascularization if clinically indicated is felt to be safe, especially in the absence of significant varices, and can improve post-LT outcomes similar to those in LT candidates without significant CAD[41,42]. Treatment of thrombocytopenia and coagulopathy as well as minimal adequate sheath sizing may reduce vascular and bleeding complication rates to those observed patients without ESLD[43]. In general, bare metal stents are usually favored in this population to minimize duration of dual antiplatelet therapy and intendant bleeding risks. In select patients, coronary artery bypass grafting may also be performed prior to LT[44]. It has been suggested that advanced age, female gender, and the presence of clinical heart failure or ascites are predictors of mortality after coronary bypass, however these data must be interpreted with caution given low representation of cirrhotic patients in surgical study cohorts[45].

Table 1 Traditional cardiac risk factors

Positive risk factors
　Age: male ≥ 45 yr, female ≥ 55 yr or premature menopause without estrogen replacement therapy
　Family history of premature coronary disease: definite myocardial infarction or sudden death before age 55 yr in male first-degree relative and before age 65 yr in female first-degree relative
　Current cigarette smoking
　Hypertension: blood pressure > 140/90 mmHg, or an antihypertensive medication
　HDL cholesterol < 40 mg/dL (1.03 mmol/L)
Negative risk factors (subtract one risk factor if present)
　HDL cholesterol ≥ 60 mg/dL (1.55 mmol/L)

Data from Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel Ⅲ). *JAMA* 2001; 285: 2486.

Table 2 Utility of non-invasive testing for coronary artery disease detection in liver transplant candidates (using coronary angiography as the gold standard)

Ref.	Screening modality	Positive predictive value	Negative predictive value
Harinstein *et al*[16]	DSE	22	75
Donovan *et al*[18]	DSE	33	100
Williams *et al*[17]	DSE	0	86
Davidson *et al*[19]	SPECT	22	77
Aydinalp *et al*[20]	SPECT	15	100
Bhutani *et al*[23]	SPECT	23	93

DSE: Dobutamine stress echocardiography; SPECT: Single photon emission computed tomography.

CARDIOMYOPATHY AND HEART FAILURE

Changes in cardiac morphology, chronotropy, systolic/diastolic performance and vascular resistance that are commonly seen in the setting of cirrhosis can compromise a patient's ability to handle the hemodynamic stresses of LT. Specifically, ELSD has been associated with chamber enlargement and hypertrophy, decreased beta-agonist transduction with bradycardia and decreased chronotropic competence, high cardiac output +/- left ventricular outflow tract obstruction, and a milieu of circulating inflammatory mediators with cardiodepressant and systemic vasodilatory properties (low SVR)[46-52]. The term cirrhotic cardiomyopathy has been applied to these alterations in normal cardiovascular physiology, and such derangements can influence ventricular response to the sudden increase in preload that immediately follows transplant graft reperfusion[53-55]. Close monitoring and optimization of cardiac function is imperative to minimize post-transplant congestion and heart failure.

Common echocardiographic features of cirrhotic cardiomyopathy include hypertrophy out of proportion to hemodynamic load, impaired diastolic relaxation, and decreased contractile reserve[56-58]. Sudden increase in preload and gradual normalization of afterload post-transplant can unmask these cardiomyopathic features to produce overt heart failure[59-62]. Preoperative features predictive of clinically significant systolic heart failure after LT include elevated right-heart and pulmonary artery pressures prior to transplant[63]. In addition, the magnitude of early hemodynamic compromise (as measured by cardiac index and oxygen delivery within the first 12 h of transplant) has been correlated with risk of multiorgan failure and mortality[64]. With aggressive supportive management, the pathophysiologic features of cirrhotic cardiomyopathy may improve with LT over time[65]. Preoperative evaluation with transthoracic Doppler echocardiography can help identify those LT candidates at greatest risk for developing clinically significant heart failure syndromes postoperatively, in order to allow optimization of volume status and heart failure symptoms prior to LT, as well as planned aggressive management of heart fail-

ure after transplantation. Beta blockers, angiotensin converting enzyme inhibitors (ACE-i) and aldosterone antagonists are generally well tolerated post-LT, and should be continued in the absence of contraindications[66]. Study of beta blockers in cirrhosis suggests that carvedilol may best improve portal hypertension and hepatic venous pressure gradients *via* decreased splanchnic blood flow and decreased portocollateral resistance[67-69]. Dose adjustments of ACE-i and aldosterone antagonists may be required in the face of renal dysfunction with calcineurin inhibitors.

Resting transthoracic echocardiography is also useful for the identification and quantification of left ventricular outflow tract obstruction (LVOTO). LVOTO may be primarily functional (secondary to the high-flow state of ESLD) or primarily mechanical (secondary to obstructive hypertrophy). In either case, the risk for intraoperative hypotension is increased, especially in those LT candidates with resting LVOTO > 36 mmHg[70]. Careful preoperative evaluation is required to allow appropriate adjustment of anesthetic strategy in those at risk. Anesthetic strategies that avoid tachycardia, minimize preload depletion and limit inotropy are preferred in the setting of hemodynamically significant LVOTO[71-74]. Marked septal hypertrophy leading to symptomatic primary mechanical LVOTO may require invasive management for alcohol septal ablation if the degree of LVOTO would prohibit LT in an otherwise appropriate surgical candidate[75,76].

PULMONARY HEART DISEASE

Pulmonary heart disease is prevalent in LT candidates and is prognostic of postoperative outcomes. Vascular dilation in the pulmonary bed with intrapulmonary right-to-left shunting, ventilation-perfusion mismatch and hypoxemia is common in ESLD (termed hepatopulmonary syndrome), and does not portend worse outcomes with LT[77]. Pulmonary pressures and pulmonary vascular resistance are not markedly elevated in hepatopulmonary syndrome, and its pathophysiologic features may in fact correct post-LT. In contrast, some LT candidates with portal hypertension can develop progressive pulmonary vascular constriction and remodeling with elevated pulmonary vascular resistance, a condition termed portopulmonary hypertension (POPH)[77,78]. POPH is estimated to be present in approximately 5%-10% of all LT candidates[79]. Extent of vascular remodeling in POPH is associated with increased mortality post-LT, with 50% mortality among those with POPH and mean pulmonary artery pressure (mPAP) 35-50 mmHg and near 100% mortality among those with POPH and mPAP > 50 mmHg[80,81]. Advanced untreated POPH with mPAP $\geq$ 35 mmHg, therefore, is considered a contraindication for LT. Aggressive screening and early referral to pulmonary hypertension specialists, ideally when right ventricular function is still preserved, may allow sufficient lowering of pulmonary pressures to allow LT[82-85].

Diagnosis of POPH should not be made on the basis of elevated pulmonary pressures alone. Baseline preoperative transthoracic echocardiography should be the initial screening strategy for the identification of PH in this population. A diagnosis of mild to moderate PH based on the estimation of the pulmonary artery systolic pressure on echocardiography has not been associated with worse outcomes. Thus for cases of worse PH, further assessment is warranted. Volume overload, left ventricular failure and high cardiac output can all contribute to elevated pulmonary pressures without conferring the same degree of perioperative risk. If there is evidence of right ventricular dysfunction or pulmonary hypertension on transthoracic echocardiography, the patient should be referred for invasive hemodynamics to differentiate pulmonary arterial from pulmonary venous hypertension. Accurate diagnosis of prohibitive POPH can only be made if mPAP is ⩾ 35 mmHg and pulmonary vascular resistance is > 3 Woods units in the setting of a normal pulmonary capillary wedge pressure (PCWP) of < 15 mmHg. If PCWP is elevated, repeat invasive hemodynamics are warranted after appropriate diuresis or volume management as clinically tolerated. Finally, clinical management of underlying lung disease and other potentially reversible contributors to pulmonary hypertension should not be neglected.

Structural heart disease is prevalent in the general population, and atrial septal defects (ASD) or patent foramen ovalae (PFO) may also be found in LT candidates. ASD may be associated with shunt physiology and potential alterations in pulmonary vascular resistance over the long-term, theoretically contributing to risk of right-heart failure after transplant. The presence of PFO prior to LT has not been associated with worse outcomes in case series[86]. Existing data does not support excluding patients for transplant consideration based upon the presence of ASD or PFO[87].

CONCLUSION

CAD, cirrhotic cardiomyopathy and pulmonary heart disease are among the more common cardiovascular maladies affecting patients with ESLD. When epicardial coronary atherosclerosis is felt to prohibit LT, revascularization with either CABG surgery or PCI should be considered. In general, percutaneous revascularization is a safe and effective therapy for obstructive CAD among LT candidates, and is valuable in optimizing otherwise suitable surgical candidates to allow downstream transplantation. During PCI, bare metal stents are generally preferred to minimize duration of dual antiplatelet therapy and bleeding risk. The pathophysiologic features of cirrhotic cardiomyopathy may be unmasked by changes in preload and afterload with LT, and should therefore prompt aggressive volume and hemodynamic management as clinically tolerated prior to transplantation, with continued close monitoring and therapy throughout the perioperative period. The presence of LVOTO > 35

mmHg may warrant adjustment of anesthetic strategy to optimize intraoperative volume status to maintain preload, heart rate to maintain diastolic filling and to avoid excessive inotropy. Portopulmonary hypertension (pulmonary vascular remodeling with elevated resistance) is associated with risk of fulminant right-heart failure and increased perioperative mortality, especially with mPAP ⩾ 35 mmHg. We recommend invasive hemodynamic assessment in all LT candidates with suggestion of right ventricular dysfunction and/or at least moderate pulmonary hypertension by echocardiography. Patients with volume overload (PCWP > 15 mmHg) and/or other reversible etiologies of pulmonary hypertension should be aggressively treated for these etiologies, with repeat assessment of pulmonary hemodynamics once euvolemic and better clinically compensated. Patients meeting hemodynamic criteria for moderate to severe POPH as detailed above should have early referral to a pulmonary hypertension specialist for advanced medical and intraoperative therapies to facilitate consideration for LT.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Cytomegalovirus infection in liver transplant recipients: Updates on clinical management

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Abstract

Cytomegalovirus (CMV) infection is a common complication after liver transplantation, and it is associated with multiple direct and indirect effects. Management of CMV infection and disease has evolved over the years, and clinical guidelines have been recently updated. Universal antiviral prophylaxis and a pre-emptive treatment strategy are options for prevention. A currently-recruiting randomized clinical trial is comparing the efficacy and safety of the two prevention strategies in the highest risk D+R- liver recipients. Drug-resistant CMV infection remains uncommon but is now increasing in incidence. This highlights the currently limited therapeutic options, and the need for novel drug discoveries. Immunotherapy and antiviral drugs with novel mechanisms of action are being investigated, including letermovir (AIC246) and brincidofovir (CMX001). This article reviews the current state of CMV management after liver transplantation, including the updated practice guidelines, and summarizes the data on investigational drugs and vaccines in clinical development.

Key words: Cytomegalovirus; Liver transplantation; Valganciclovir; Prophylaxis; Treatment; Resistance; Investigational; Letermovir; Brincidofovir

Core tip: In this article, the authors review the current literature of cytomegalovirus (CMV) infection after liver transplantation, including the approaches to diagnosis, prevention and treatment. The review highlights the pros and cons of the prophylaxis *vs* pre-emptive prevention strategies, especially in the highest risk D+/R- population. Treatment of CMV infection in liver transplant patients is discussed in addition to management of CMV resistance, with detailed discussion of recently updated clinical CMV management guidelines. Finally, the future management of CMV in liver transplant recipients relies on new drug discoveries, and the authors describe multiple investigational drugs and vaccines in clinical trials.

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INTRODUCTION

Cytomegalovirus (CMV) is a ubiquitous double-stranded DNA virus also known as human herpesvirus 5 (HHV-5). CMV seroprevalence has been reported to be around 60% in the United States[1-4]; higher prevalence has been noted in developing countries[2] and among high risk

patients, including patients infected with human immunodeficiency virus (HIV)[5]. Variable rates of prevalence have been reported among ethnic groups in the United States, with greater prevalence in the non-Hispanic blacks and Mexican-Americans[1]. CMV is an important cause of clinical disease in neonates and immunocompromised patients, and it appears to also be associated with poor outcome in critically ill immunocompetent patients[6]. All members of the Herpesviridae family establish latency in infected cells, with the life-long potential for reactivation and production of infective viral particles[7,8]. Primary CMV infection in immunocompetent hosts is usually asymptomatic or a nonspecific viral illness followed by latency; however immunocompromised hosts are at higher risk for developing serious primary infection or reactivation. CMV remains latent primarily in lymphoid organs and myeloid cells[8]. It can be transmitted by exposure to body fluids including saliva, semen, blood and breast milk[2], and can also be transmitted *via* transplantation of solid organs including heart, kidney, lungs and liver[7]. CMV can be a significant problem for transplantation, as it can increase the predisposition to develop serious infections, and increases the risk of allograft rejection and mortality[9]. In this article, we highlight the impact of CMV on liver transplantation and reviews recent advances in the prevention, diagnosis, and treatment of CMV in liver transplant recipients.

CLINICAL IMPACT OF CMV

CMV disease vs infection

CMV remains as one of the most important infectious complications after liver transplantation[3,10]. Multiple risk factors are associated with its occurrence, but most notable among them are the donor/recipient CMV serostatus and the severity of pharmacologic immunosuppression[1]. CMV infection represents the presence of the virus, as indicated by the detection of viral proteins or nucleic acids in body fluids or tissue samples, regardless of clinical symptoms[11]; the presence of any clinical symptoms in patients with CMV infection is termed CMV disease. CMV disease in immunocompromised patients may affect one or multiple organs, but may also have atypical presentations, requiring close monitoring and high index of suspicion in transplant patients[12].

Direct effects

The direct effects of CMV after liver transplantation can be categorized as CMV syndrome or tissue invasive disease (Table 1). CMV syndrome is the term for the clinical illness characterized by fever, constitutional symptoms, and myelosuppression in the presence of CMV infection[7,8,11]; this accounts for the majority of CMV disease cases in liver transplant recipients. In addition, CMV can invade end-organs and cause tissue-invasive disease. The most common organ affected is the gastrointestinal tract, causing esophagitis, gastritis, enteritis, or colitis[7]. There is the predisposition for the transplanted allograft to

Table 1 Direct and indirect clinical effects of cytomegalovirus in transplant recipients

Direct clinical effects	Indirect clinical effects
CMV syndrome	Acute allograft rejection
Fever > 38 ℃ for 2/4 d	Chronic allograft rejection
Malaise	Allograft failure
Myelosuppression	Vanishing duct syndrome/ductopenia
Tissue-invasive CMV disease[1]	Allograft hepatitis and fibrosis
Gastrointestinal disease	
(entire gastrointestinal tract	Vascular thrombosis
can be affected)	Opportunistic and other infections
Hepatitis	Fungal (Aspergillus, Pneumocystis)
Pneumonitis	Bacterial (Nocardia)
Retinitis	Viral (HHV-6, HHV-7, EBV)
CNS disease	Hepatitis C virus recurrence
Carditis	EBV associated PTLD
Mortality	Mortality

[1]Can affect any organ, listed are most common organs affected. PTLD: Post-transplant lymphoproliferative disorder; HHV: Human herpes virus; EBV: Epstein-Barr virus; CMV: Cytomegalovirus.

develop CMV infection in solid organ transplant (SOT) recipients, likely secondary to an abnormal allograft immune response[13]. It is therefore not uncommon for liver transplant recipients to develop CMV hepatitis[14]. Allograft invasion by CMV is likely the result of viral reactivation in the transplanted liver allograft that contains donor-transmitted virus[2,9].

Indirect effects

Indirect effects of CMV include inflammatory cytokine-mediated acute and/or chronic graft rejection, decreased graft survival, and increased patient mortality[8,15,16]. CMV can also have an immunomodulatory effect in liver recipients, further enhancing the immunosuppression and predisposition to opportunistic infections with bacteria, fungi or other viruses, including EBV-related post-transplant lymphoproliferative disorder and accelerated HCV recurrence[8,13,15]. CMV disease has been found to be an independent risk factor in the development of invasive fungal infections in liver transplant recipients[10].

RISK FACTORS

The most important clinical predictor for the development of CMV infection and disease after liver transplantation is the CMV serostatus of the donor and the recipient[13,17-19]. Accordingly, prior to transplantation, donors and recipients are screened for CMV IgG antibodies to accurately stratify patients into risk groups for CMV disease[13]. The risk for serious CMV disease is highest for primary infections in CMV seronegative recipients receiving CMV seropositive allografts (CMV D+/R- mismatch)[13,19]. One study reported an almost universal CMV infection among D+/R- liver transplant recipients[20]. In contrast, double negative donor-recipient combinations (CMV D-/R-) have the lowest risk for CMV disease after liver transplantation[21]. Only about 1%-2% of this CMV

D-/R- transplant population will develop CMV disease during the first year after liver transplantation, either as a result of natural transmission or through blood transfusion. In order to preserve this low CMV risk, these patients must receive leukoreduced and/or CMV antibody negative blood products[13,20].

The intensity of the immunosuppressive regimen used in the post-transplant period plays an important role in determining the patient's overall immune status[20,22]. Patients given antilymphocyte antibodies (such as thymoglobulin, alemtuzumab, among others) are significantly increased risk of CMV infection and disease compared to their counterparts not receiving this therapy, whether these agents are used as induction or anti-rejection therapy[13]. After these therapies are administered, there is a systemic release of tumor-necrosis factor-alpha, which is a potent transactivator of latent CMV[23]. The incidence rates of CMV infection and disease in liver transplant recipients are higher during treatment for allograft rejection, likely due to the accelerated inflammatory state[13,21], especially among those who did not receive CMV prophylaxis[23].

Certain co-infections also increase the risk for developing CMV infection and disease, especially HHV6 and HHV7[13,20,24]. There are also reports correlating CMV disease risk with lower model for end stage liver disease scores, lower total bilirubin, and higher operating time, but these findings have been inconsistent among different studies[25].

Deficiencies in innate and cell-mediated adaptive immunity have been associated with CMV disease after liver transplantation. Specifically, functional polymorphisms in Toll-like receptors, which are important receptors that recognizes CMV, has been associated with increased risk of CMV disease after liver transplantation[26]. Homozygosity for Toll-like receptor 2 R753Q single nucleotide polymorphism is a marker for tissue-invasive CMV disease after liver transplantation, independent of other traditional risk factors[27]. Likewise, liver transplant recipients who are deficient in CMV-specific T cells, as indicated by undetectable or low levels of interferon-gamma during stimulation with CMV antigens, are at significantly higher risk of CMV disease.

DIAGNOSIS

Several methods are used for the diagnosis of CMV infection after transplantation. Among them, the most commonly used technique is nucleic acid testing (NAT), which is often performed using polymerase chain reaction (PCR). Using this approach, CMV viral load has been used for various indications, including (1) rapid diagnosis of CMV infection; (2) prognostication of the severity of infection; (3) monitoring for antiviral efficacy; and (4) assessing the risk of relapse[20,28]. Real-time NAT are faster technologies with rapid turn-around time that will confirm the suspicion of CMV disease within hours of testing[28]. Its quantitative ability allows for assessing the severity of infection (higher viral load is associated with higher likelihood of severe disease). Serial monitoring will also allow for assessment of viral load rise (associated with disease progression) or decline (associated with antiviral response). Indeed, the rate of change of viral load over time is as important as the absolute viral load values in CMV disease assessment and prognostication[13,20,28]. CMV NAT is often performed on blood samples, and there is ongoing debate as to which compartment of the blood is ideal for CMV NAT. Whole blood samples often allow for the detection of viremia earlier and for longer periods than plasma samples[29]; whether this is better for diagnostic purposes is still debated. The only FDA approved CMV NAT assay in the US detects the virus on plasma samples[28]. It is important to point out that a negative CMV NAT in blood samples does not completely rule out the presence of compartmentalized and localized tissue-invasive CMV cases (such as some cases of reactivation CMV gastrointestinal disease, and CMV retinitis)[13].

Until recently, CMV NAT assays are not directly comparable. Differences in assay design, platform, target, calibrators, and samples (among others) have limited direct comparison and portability of viral load results from one assay to another. Accordingly, there were no widely accepted viral load thresholds that can be used for CMV disease management[30,31]. To address this, a World Health Organization (WHO) International Standard was developed to which assays can be calibrated for viral load reporting[32,33]. Using an assay that has been calibrated to this WHO standard, one study had suggested a viral load of 3983 IU/mL (2600 copies/mL), with a 99.6% negative predictive value (89.9% sensitivity and 88.9% specificity) as an appropriate cut-off for initiating treatment in CMV-seropositive SOT recipients[31]. Another study reported that suppression of the viral load to < 137 IU is associated with faster clinical CMV disease resolution. Whether this will also result in lower rate of CMV disease relapse, and what viral load threshold can be used for various indication are now being investigated in the clinical setting.

The gold standard for diagnosis of tissue invasive CMV disease remains the demonstration of CMV pathology in a biopsy specimen from the involved organ. CMV can be demonstrated in the biopsy specimen using histology (such as demonstration of CMV inclusion bodies), immunohistochemical identification of CMV antigens, in-situ DNA hybridization or (less preferred) CMV culture[28]. Serology to demonstrate CMV IgG in the blood of transplant recipients is not recommended for the diagnosis of acute CMV infection after transplantation. Liver transplant recipients may have delayed and impaired ability to mount antibody production, thereby making serology not as reliable in diagnosis of acute infection after transplantation.

Detection of CMV pp65 using fluorescent methods on infected peripheral blood leukocytes is another method for the rapid diagnosis and surveillance of CMV infec-

Table 2 Comparison of antiviral prophylaxis and pre-emptive strategies for cytomegalovirus prevention in liver transplant patients

Prevention characteristics	Prophylaxis strategy	Pre-emptive strategy
CMV disease	Very effective at preventing CMV infection and disease	Effective to prevent CMV disease; does not prevent CMV infection
Late-onset CMV disease	Higher risk of late and very-late onset CMV disease	Reduces incidence of late onset CMV disease
Ideal treatment population	CMV D+R- are highest risk patients	CMV R+ patients
Logistics of strategy	Logistically more feasible, but still requires frequent monitoring of adverse effects	Requires weekly viral load testing; standardized viral load thresholds still being investigated
Cost	Higher drug costs; lower laboratory/monitoring costs	Higher laboratory/monitoring costs; lower drug costs
Safety/adverse effects	More frequent adverse effects such as myelosuppression due to longer treatment periods	Shorter treatment periods; fewer toxicities
Indirect CMV effects	Better evidence showing reduction of graft rejection, improved graft survival, opportunistic infections	Limited evidence overall, but may not reduce indirect effects
Effect on mortality	Reduces mortality from CMV disease	Limited evidence regarding mortality reduction
CMV resistance	More common compared to pre-emptive strategy	Some evidence regarding effect on resistance but overall uncommon

CMV: Cytomegalovirus.

tion in transplant recipients. Its utility has been declining with the rise in molecular methods such as CMV NAT. In some studies, the CMV pp65 had comparable sensitivity to CMV PCR, and higher sensitivity compared to culture-based methods[13]. However, the pp65 antigenemia assay has lower rates of detection in the lower viral load ranges[30,34]. Additionally, although the specificity of the antigenemia assay and CMV NAT are comparable, the CMV NAT have a higher negative and positive predictive value and much higher sensitivity than the pp65 assay in SOT recipients[30]. Moreover, the pp65 antigenemia assay is time sensitive: samples must be obtained and tested in 8 h. There is also significant biological variation, and its accuracy decreases in leukopenic patients with absolute neutrophil counts less than 1000/mm^3 (since pp65 antigen is detected on leukocyte populations)[28,34].

PREVENTION

In the absence of any prevention strategy, CMV infection (36%-100%) and disease (11%-72%) can be expected to occur within the first 3-4 mo after liver transplantation[2,14,35]. This may be subclinical or may present clinically as CMV syndrome or end organ disease[14]. In a large cohort of liver transplant patients, there was an independent association between CMV infection or disease within the first year of liver transplantation and the composite outcome of allograft loss or mortality (RR = 3.04, 95%CI: 1.56-5.92, P = 0.001)[36].

Prevention of CMV infection and disease in liver transplant recipients is therefore a priority in post-transplant management. This can be accomplished either with antiviral prophylaxis or pre-emptive therapy, and the strategies vary according to institution. Universal prophylaxis provides antiviral therapy to all patients at risk for CMV infection after liver transplantation, while the preemptive strategy involves frequent viral load monitoring and treatment at the early stages of CMV infection before symptomatic disease develops[13]. In a retrospective analysis of two liver transplant cohorts receiving prophylaxis or preemptive therapy, CMV viremia expectedly occurred within the first 3 mo after liver transplantation

in the preemptive strategy group, while this occurred during months 3-6 among patients who received 3 mo of antiviral prophylaxis[37]. Each of the two approaches has advantages and disadvantages as shown in Table 2 but the prophylaxis strategy has been preferred among the majority of transplant programs in the United States, partly due to better long-term outcome and for logistic reasons[38]. The major disadvantage of antiviral prophylaxis is the occurrence of late-onset CMV disease. To reduce this complication, some institutions have adopted a hybrid approach whereby patients who receive antiviral prophylaxis initially are subjected to a preemptive strategy during the high-risk period after antiviral prophylaxis[20].

Antiviral prophylaxis

CMV prophylaxis is usually given to all patients at-risk during the initial 3-6 mo after liver transplantation. Additionally, antiviral prophylaxis is given to patients receiving lymphocyte-depleting therapy for acute allograft rejection[20]. The recommendations for CMV prophylaxis vary depending on the serostatus of the donor and recipient. CMV D+/R- liver recipients are recommended to receive 3-6 mo of CMV prophylaxis, while 3 mo of prophylaxis may be sufficient for the CMV-seropositive liver recipients. Most commonly, CMV prophylaxis is with the use of valganciclovir (dose 900 mg daily, adjusted based on renal function). Alternative agents are oral ganciclovir (3 g per day) or intravenous ganciclovir (5-mg/kg daily)[13]. Because of the higher rates of tissue-invasive CMV disease in liver transplant recipients who received valganciclovir compared to oral ganciclovir prophylaxis, the US FDA did not approve the use of valganciclovir for CMV prophylaxis in liver transplant recipients. Notwithstanding this statement, multiple experts recommend the use of valganciclovir for prevention of CMV in liver transplant patients, and it is subsequently the preferred therapy in a large majority (> 70%) of transplant centers in the United States[38]. A more recent study confirmed the increased incidence of late-onset CMV disease in liver recipients[39]. However, valganciclovir has superior bioavailability compared to oral ganciclovir (50%-60% compared to 6%-9%)[13,40]. A number of more recent retrospective

studies found no significant difference in incidence of CMV disease in liver transplant patients treated with oral ganciclovir *vs* valganciclovir[41,42].

Due to the major adverse effect of leukopenia and the high cost of the drug, low-dose valganciclovir dosing has been proposed. In a recent review, there remains a high risk of CMV disease in liver transplant patients, despite the use of valganciclovir prophylaxis at a dose of 900-mg daily[43]. In a large meta-analysis, the efficacy of valganciclovir 450-mg was equivalent to valganciclovir 900-mg dosing, and the low-dose program was associated with lower incidence of leukopenia and fewer instances of CMV disease or graft rejection[44]. It is possible that the low dose valganciclovir would allow for a low level exposure of the immune system to CMV antigens, thereby allowing for T cells to mount an immune response[44]; this however remains speculative. In an earlier retrospective study of liver transplant recipients, the efficacy of valganciclovir at 450-mg dosing was similar to oral ganciclovir[40].

One of the major drawbacks to antiviral prophylaxis is the occurrence of late-onset CMV disease, which is the term for CMV disease cases that occur soon after the completion of universal prophylaxis. Among liver transplant recipients who receive 3 mo of antiviral prophylaxis, late-onset CMV disease would typically occur at about 3-6 mo after transplantation[14,45]. The incidence of late-onset CMV disease has been reported to vary between 17%-37% depending on the length of valganciclovir prophylaxis[45-47]. Late-onset CMV disease is almost exclusively a condition that occurs in CMV D+/R- SOT recipients who received antiviral prophylaxis, and rarely is observed in CMV-seropositive transplant recipients. To address the issue of late-onset CMV disease, many have suggested longer periods of CMV prophylaxis[45,46]. In a study of kidney transplant recipients (The IMPACT study), antiviral prophylaxis for 200 d was associated with lower incidence of CMV disease compared to 100 d of prophylaxis, and that the number needed to treat to avoid one additional CMV disease diagnosis up to 12 mo posttransplant was 5[45,46]. Fewer patients in the 200 d group developed CMV disease at 6, 9, 12 and 24 mo (7.1%, 14.2%, 16.1% and 21.3% respectively) compared to the 100 d group at 6, 9, 12 and 24 mo (31.3%, 35%, 36.8% and 38.7% respectively); *P* value < 0.0001 in all cases[45,46]. Additionally, longer prophylaxis was associated with fewer opportunistic infections than standard prophylaxis (12.9% compared with 27%, *P* = 0.001)[45,46]. While this was conducted in kidney transplant recipients, many centers have extrapolated the findings to high risk CMV D+/R- liver transplant recipients.

Preemptive therapy
Preemptive therapy is the approach wherein antiviral therapy is provided to liver transplant recipients with low-level asymptomatic CMV infection. The mainstay of preemptive therapy is close laboratory monitoring of the at-risk patients and the initiation of early antiviral treatment

when a viral load threshold is reached, so that the infection does not progress to CMV disease[13,14]. Typically, the preemptive approach involves weekly CMV monitoring with the use of pp65 antigenemia assay or CMV NAT by PCR, for at least 12 wk after liver transplantation[13,14]. Once the virus is detected, antiviral treatment can be initiated either with oral valganciclovir (900 mg twice daily) or IV ganciclovir (5 mg/kg every 12 h). Antiviral treatment is continued until the virus is no longer detected in the blood[13,14]. Many transplant centers are not comfortable with using this approach in the highest risk CMV D+/R- group because the rapid viral replication dynamics in this high-risk patient may lead to the inability to detect CMV soon enough for the initiation of effective antiviral treatment. Many studies however have demonstrated the efficacy of preemptive therapy in moderate-risk groups such as CMV R+ liver transplant recipients[13,14]. In a study of liver transplant population (a third were D+/R-), the use of preemptive therapy guided by CMV NAT by PCR was associated with CMV disease rates < 1% at one year and < 2% overall, and the inclusion of CMV D+/R- in this study suggests that it is also feasible even in high-risk patient groups[48].

The preemptive strategy is advantageous in terms of reducing cost and drug toxicity if used in recipients who are at lower risk for developing CMV disease. Given the advantages of the preemptive approach in cost-effectiveness and adverse effect reduction, more studies need to be designed to test this approach in the highest risk D+/R- patients for whom the prophylaxis strategy is currently recommended. One ongoing phase 4 randomized, controlled study is performing direct head to head comparisons of the prophylaxis and preemptive strategies in D+/R- liver transplant patients[49], and hopefully the results of this and subsequent trials should help to answer this question (ClinicalTrials.gov Identifier: NCT01552369).

TREATMENT
The standard of care for the treatment of CMV disease is IV ganciclovir, at 5 mg/kg twice daily, or valganciclovir, at 900-mg orally twice daily[13]. IV ganciclovir administration requires intravenous access, the potential need for inpatient hospitalization, and may be complicated by catheter-related bacterial or fungal infections. Oral valganciclovir has excellent bioavailability that provides systemic ganciclovir levels comparable to IV ganciclovir, but it relies on efficient absorption through the gastrointestinal tract. Both valganciclovir and intravenous ganciclovir have been demonstrated to be efficacious for the treatment of mild to moderate CMV disease and asymptomatic CMV infection in SOT recipients. In the VICTOR study, the safety and non-inferiority of oral valganciclovir at 900 mg twice daily was demonstrated in comparison to IV ganciclovir for the treatment of CMV disease in SOT patients, 7% of whom were liver transplant recipients[50]. Oral valganciclovir administration resulted in almost

identical mean times to clinical resolution of disease as IV ganciclovir, with no differences in the incidence of graft rejection or adverse effects at the end of the 21-d treatment period[50]. One year follow up of these patients revealed no difference in long-term treatment outcomes in patients treated with oral valganciclovir compared to IV ganciclovir. Guidelines therefore recommend the use of oral valganciclovir for treatment of mild-to-moderate CMV disease, but not in life-threatening CMV disease cases, those with very high viral load, those patients with poor oral absorption, and those with questionable compliance with medications. In such cases, IV ganciclovir would be more appropriate[51]. Oral ganciclovir, acyclovir or valacyclovir should not be used for treatment of CMV disease[51]. IV foscarnet and cidofovir should not be used as first-line drugs for the treatment of CMV disease due to their toxicity profiles, but are considered alternative agents for treatment of ganciclovir-resistant CMV.

The duration of treatment of CMV disease should be guided by viral load monitoring. Previous studies have demonstrated that the greatest predictor of clinical relapse was the persistence of CMV viremia at the end of treatment[52]. Indeed, suppression of CMV viral load to less than 137 IU/mL was associated with resolution of clinical disease. Thus, it is recommended that transplant recipients with CMV disease should undergo weekly viral load monitoring, which will guide the duration of treatment. Guidelines currently recommend antiviral treatment until two weekly negative CMV PCR's are demonstrated. Patients treated with valganciclovir and intravenous ganciclovir should also be monitored closely for adverse effects, which include leukopenia[51].

In addition to antiviral drugs, SOT recipients with CMV disease should be assessed for the intensity of immunosuppression. In theory, CMV is an opportunistic pathogen and its onset is often correlated with a more severe intensity of immune dysfunction. Accordingly, one should consider reducing the dose of immunosuppressive therapy in patients with CMV disease, especially if the illness is severe. This should however be done cautiously since drastic and precipitous reduction in immunosuppression may precipitate allograft rejection.

Antiviral resistance

Ganciclovir resistant CMV is still uncommon in the general SOT population, although it has been described to occur in up to 7% of high-risk SOT recipients who previously received prolonged ganciclovir prophylaxis[53]. Prolonged use of ganciclovir predisposes to the development of CMV resistance[51]. It should be suspected if viral loads do not decline despite effective antiviral therapy for at least 2-3 wk, if clinical symptoms recur, or if viral load is not suppressed to undetectable levels despite prolonged effective therapy[51,54].

To become an active drug, ganciclovir must be phosphorylated to ganciclovir triphosphate *via* three enzymatic steps; the initial phosphorylation is catalyzed by *UL97*-encoded viral kinase[54]. The ganciclovir triphosphate subsequently incorporates competitively into the DNA *via* the *UL54* DNA polymerase and stops viral replication[54]. Cidofovir and foscarnet do not require the initial *UL97*-catalyzed phosphorylation, but they also act on *UL54* DNA polymerase to terminate viral replication. The primary mechanism for CMV resistance to ganciclovir is due to mutations of the *UL97* phosphotransferase gene which is responsible for the initial ganciclovir monophosphorylation step[55]. Less commonly, mutations in *UL54* may occur, and this may result in cross-resistance among ganciclovir, foscarnet and cidofovir.

The CMV D+/R- transplant recipients are at highest risk of drug resistance with an incidence of 5%-10%; rates of resistance vary depending on the organ transplanted, with greater incidence in lung and pancreas recipients[54,55]. While traditionally considered to be at a lower risk, transplant patients on preemptive therapy have also been demonstrated to be at risk of ganciclovir-resistant CMV disease, especially if preemptive therapy is prolonged or when oral preemptive therapy is started when the viral load is initially high[54,55]. Earlier studies described an increased incidence of resistance in patients treated with oral ganciclovir compared to valganciclovir, attributed to better viral suppression in the valganciclovir group[39,56]. More recently however, SOT recipients treated with either IV ganciclovir or oral valganciclovir have been found to have similar risk of developing ganciclovir resistance[55]. Genotypic drug assays have been developed to identify ganciclovir resistance mutations to *UL97* and *UL54* and can provide relatively rapid confirmation in cases of suspected ganciclovir resistance[51,54].

In life-threatening cases where antiviral resistance is suspected, an empiric switch in treatment, often to foscarnet, is recommended. If low-level resistance is confirmed, increasing the dose of IV ganciclovir (or switching from oral valganciclovir to IV ganciclovir) is recommended as first-line of treatment with close monitoring for myelosuppressive and nephrotoxic adverse effects[51]. In cases of high-level resistance, foscarnet is the recommended antiviral therapy[51]; since many ganciclovir resistance mutations also confer cidofovir resistance, the latter is a poor alternative[54]. Foscarnet (second line) and cidofovir (third line) are associated with significant nephrotoxicity however, and can be challenging to administer in patients with reduced renal function or electrolyte imbalances. Ganciclovir-foscarnet combination therapy has been proposed as an option for treatment of resistant CMV, but this has variable response and is no longer strongly recommended since it was associated with more toxicity without proven clinical benefits[57].

There are some data to support the use of adjunctive treatments like intravenous immunoglobulin or CMV immunoglobulin (CMV Ig), a switch to mammalian target of rapamycin inhibitors (sirolimus, everolimus), or use of leflunomide[51]. The incidence of CMV disease has been lower than expected in transplant recipients on sirolimus-containing immunosuppressive regimens[58]. Another recent study revealed that while CMV-Ig and CMV neutral-

izing antibodies may be able to reduce viral spread during initial infection, during subsequent reactivation, they are unable to stop cell-to-cell viral spread[59]. Additionally, CMV-Ig has been shown to reduce CMV related mortality in SOT patients[60].

Several drugs in clinical development have been used for prevention and treatment of CMV disease, including those caused by drug-resistant CMV. Maribavir, a benzimidazole inhibitor of the *UL97* kinase, has been shown to be a potential drug for the treatment of drug-resistant CMV. However, the clinical trials in HSCT and liver transplant recipients were disappointing and the drug was not effective for CMV infection and disease prevention. In a randomized controlled trial comparing maribavir to oral ganciclovir as prophylaxis in liver transplant patients, there were significantly fewer patients with CMV infection or disease at 3 or 6 mo when treated with ganciclovir compared to maribavir[61]. Some have speculated that the dose of maribavir chosen (100-mg twice daily) contributed to the inability to prove noninferiority, as previous phase Ⅱ trials demonstrated adequate antiviral activity at higher doses (400-mg twice daily), but higher doses were associated with significant dysgeusia[62]. Maribavir may still remain an option for treatment of multi-drug resistant CMV due to differences in resistance mechanisms[63]. New clinical trials (ClinicalTrials.gov Identifier: NCT01611974) are actively recruiting SOT and HSCT patients to investigate maribavir as a treatment for resistant or refractory CMV[64].

Another investigational agent is letermovir (AIC246) which inhibits the enzyme UL56 terminase and shows promise in early studies with antiviral efficacy against drug resistant strains and minimal cross-resistance[51,65-67]. Additionally, cyclopropavir is another novel compound that has been shown to be effective against CMV by inhibition of the *UL97* kinase[68,69]. Resistance usually involves mutations of *UL97* kinase that are different from those seen in ganciclovir resistance, therefore cyclopropavir can potentially still retain activity against ganciclovir resistant strains[68]. CMX001 (Brincidofovir) is another investigational therapy with activity against multiple DNA viruses[70]. It is a well-tolerated oral lipid-conjugate derivative prodrug of cidofovir which delivers antiviral directly to target cells before being cleaved, and with no evidence of nephrotoxicity or myelosuppression and increased potency compared to cidofovir[63,71]. Multiple clinical trials using CMX001 are ongoing, including one which preliminarily reported that CMV viremia was reduced with CMX001 use in HSCT patients with refractory CMV disease[72]. Additionally, results of the phase Ⅱ randomized, controlled, dose-escalated clinical trial presented at the 2012 BMT Tandem meetings revealed a reduction in new or progressive CMV infection in HSCT patients using higher doses of CMX001 for CMV prophylaxis[73]. The phase Ⅲ study (ClinicalTrials.gov Identifier: NCT01769170) is now recruiting participants to continue to study the safety and efficacy of CMX001 for CMV prevention in HSCT patients[74]. Resistance patterns in CMX001 would theoretically be similar to cidofovir,

however a *de novo* CMX001 resistant strain (D542E, a novel *UL54* mutation) has been generated after prolonged selection pressure *in vitro*[75]. Clinical trials of these compounds in the liver transplant population are yet to be performed.

Other drugs approved for other indications have been used off-label and anecdotally for treatment of resistant CMV. Leflunomide is an anti-inflammatory drug approved for rheumatoid arthritis that also causes inhibition of CMV viral kinases and pyrimidine synthesis[54]; however more investigations need to be conducted to identify its role in the treatment of multi-drug resistant CMV. Similarly, artesunate is an antimalarial with activity against drug resistant CMV, with proposed activity against viral kinase signaling pathways; experience with artesunate in this capacity has only been anecdotal[54].

Due to the virus' ability to evade host defenses, primary infection with CMV has not been shown to confer immunity from subsequent infections[76]. Notwithstanding this, there are efforts to develop CMV vaccine for prevention and therapy. These however remain in clinical development, and none has been subjected to phase Ⅲ clinical trials.

CONCLUSION

Despite decades of studies dedicated to the discovery of new treatment and prevention options, CMV remains the single most devastating viral infection causing morbidity and mortality in liver transplant patients[20]. It is known that contributions from both the innate and adaptive immune system are necessary for a complete immune response to CMV, but the virus has a unique ability to evade both arms, causing latency and reactivation. Multiple antiviral therapies are approved for treatment and prophylaxis of CMV infection in transplant patients, but ganciclovir (and valganciclovir) is the most commonly used antiviral drug. High-risk D+/R- liver transplant patients require a more aggressive form of prevention, which in many centers have translated to longer duration of antiviral prophylaxis. Preemptive therapy may also work, if coordinated properly. Multiple viral mutations in *UL97* and less commonly *UL54* gene contribute to CMV resistance, and the challenge in these times is to produce a reliable alternative treatment option in cases of multi drug resistant CMV. Several drugs and compounds are currently being developed in clinical trials, including a potentially effective vaccine to reduce the impact of CMV on transplantation outcomes[77].

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Recurrent hepatitis C after liver transplant

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Abstract

End stage liver disease from hepatitis C is the most common indication for liver transplantation in many parts of the world accounting for up to 40% of liver transplants. Antiviral therapy either before or after liver transplantation is challenging due to side effects and lower efficacy in patients with cirrhosis and liver transplant recipients, as well as from drug interactions with immunosuppressants. Factors that may affect recurrent hepatitis C include donor age, immunosuppression, *IL28B* genotype, cytomegalovirus infection, and metabolic syndrome. Older donor age has persistently been shown to have the greatest impact on recurrent hepatitis C. After liver transplantation, distinguishing recurrent hepatitis C from acute cellular rejection may be difficult, although the development of molecular markers may help in making the correct diagnosis. The advent of interferon free regimens with direct acting antiviral agents that include NS3/4A protease inhibitors, NS5B polymerase inhibitors and NS5A inhibitors holds great promise in improving outcomes for liver transplant candidates and recipients.

Key words: Hepatitis C; Liver transplant; Donor risk factors; Immunosuppression; Protease inhibitors; Fibrosing cholestatic hepatitis C; Acute cellular rejection; Cytomegalovirus

Core tip: Recurrent hepatitis C impacts graft and patient survival following liver transplant. Preventing aggressive hepatitis C virus (HCV) recurrence by selecting appropriate donor allografts for HCV patients and careful management of immunosuppression in the post-transplant setting remain crucial. Direct acting antiviral therapy in patients awaiting transplant may prevent HCV re-infection post-transplant and has the potential to fundamentally change the natural history of hepatitis C in liver transplant recipients.

BACKGROUND

End stage liver disease due to hepatitis C virus (HCV) infection remains a leading indication for liver transplantation (LT) worldwide. While eradication of virus prior to LT is ideal, currently available antiviral therapy for those awaiting transplant is limited by toxicities and low response rates. Viral recurrence following LT is immediate and universal. There are donor, host, and transplant related factors which increase the likelihood and severity of recurrence. Some of these factors are modifiable.

Recurrent HCV infection is associated with more rapid fibrosis progression leading to higher rates of graft loss and patient mortality compared to patients transplanted for non-HCV etiologies. Post-transplant HCV antiviral treatment is also challenging due to poor tolerability, drug-drug interactions with immunosuppressant agents, and low response rates. Re-transplantation for

allograft cirrhosis due to recurrent HCV remains controversial. This review will address pre- and post-transplant hepatitis C infection, antiviral treatment strategies, and the future role of direct acting antiviral agents.

TREATMENT OF HEPATITIS C IN CIRRHOTIC PATIENTS AWAITING LIVER TRANSPLANTATION

The most effective way to avoid post-LT HCV recurrence is to eradicate virus prior to transplant. Treating patients who are awaiting transplant, however, has been difficult with currently available regimens due to side effects and limited efficacy. The best-suited treatment candidates are those with compensated cirrhosis [Child-Pugh class A, model for end-stage liver disease (MELD) < 13] or those with hepatocellular carcinoma (HCC) as the primary indication for transplant. Unfortunately, this scenario is not the norm for most liver transplant candidates who have advanced liver failure.

Several published studies have examined the role of standard or pegylated interferon (PEG-IFN) with or without ribavirin (RBV) before liver transplantation in cirrhotic patients (Table 1). One large single-center study treated 124 patients (mean MELD score 11.0 ± 3.7) with a low accelerating dosage regimen (LADR) of antiviral therapy[1]. Most patients were treated with interferon alfa-2b and RBV; PEG-IFN and RBV was reserved for use in the retreatment of 15 patients. Treatment was initiated with half doses of interferon and RBV. Dose adjustments were made every 2 wk to reach maximally tolerated or target standard doses. Sustained virologic response (SVR) was achieved in 13% of patients with genotype 1 HCV and 50% of patients with non-1 genotypes. Twelve of 15 patients who were HCV-RNA negative prior to LT remained virus negative 6 mo or more after transplant. Fifteen patients experienced 22 serious adverse events (SAEs) including infection, diabetes mellitus, severe thrombocytopenia, and venous thromboembolism[1]. This study showed an acceptable SVR for non-genotype 1 patients with advanced disease, but highlighted the importance of following these patients closely while on treatment, preferably with the safety net of liver transplantation in place.

Carrión *et al*[2] published the first study using PEG-IFN and RBV in hepatitis C patients awaiting LT. Fifty-one patients with HCV cirrhosis (mean MELD 12) were matched to 51 untreated controls. While the on-treatment virologic response was 47%, 29% were HCV RNA negative at the time of LT, and only 20% achieved a SVR after LT. Early virologic response and non-1 genotype were the strongest predictors of viral clearance. Child-Pugh B/C patients had a particularly high incidence of bacterial infection with bacteremia and spontaneous bacterial peritonitis. Three control and 12 treated patients developed 3 and 19 bacterial infections, respectively. The authors recommended using caution when treating those with decompensated disease who are not on prophylactic

Table 1 Treatment of hepatitis C virus cirrhosis with interferon and ribavirin

Ref.	*n*	Child score	Treatment	Mean treatment duration (mo)	End of treatment response/post-LT SVR
Everson *et al*[1]	124	7	IFN and RBV	6-12	46%/30%
Carrion *et al*[2]	51	88% A or B	PEG-IFN and RBV	3	29%/20%
Everson *et al*[3]	63	7	PEG-IFN and RBV	4	59%/25%

LT: Liver transplantation; PEG-IFN: Pegylated-interferon; RBV: Ribavirin; IFN: Interferon; SVR: Sustained viral response rate.

antibiotics[2].

A randomized controlled trial (LADR-A2ALL) evaluated PEG-IFN and RBV in a cohort of 79 patients with advanced HCV who were candidates for adult living donor LT[3]. Patients with genotypes 1/4/6 (*n* = 44/2/1) were randomized 2:1 to treatment or untreated control; HCV genotypes 2/3 (*n* = 32) were assigned to treatment. Two groups of adult patients were included: those who had a potential living donor and those with HCC eligible for a MELD upgrade; the average native MELD score was 12 in both the treated and control groups. Pre-transplant treatment achieved post-transplant viral clearance (pTVR = negative viral load 12 wk after transplant) in 25% of patients. The only factor predictive of pTVR was longer duration of pre-transplant treatment. More specifically, pTVR was 0%, 18%, and 50% in patients treated for < 8, 8-16, and > 16 wk, respectively. SAEs occurred during the course of treatment and the number of SAEs per patient was higher in the treated group (2.7 *vs* 1.3, *P* = 0.003). In fact, after several serious infections, the authors broadened the use of antibiotic prophylaxis to include patients with a current or past history of ascites.

Triple therapy using first generation HCV NS3/4A protease inhibitors for genotype 1 HCV led to improved SVR rates overall. However, it's important to note that relatively few patients with advanced fibrosis were included in the phase 3 registration trials of telaprevir and boceprevir[4,5]. Patients with advanced fibrosis and prior relapse to interferon therapy actually did better than treatment naïve patients with advanced fibrosis (Table 2)[6,7]. Unfortunately, the initial enthusiasm to treat those chronic hepatitis C patients with cirrhosis with first generation triple therapy was tempered by an unfavorable side effect profile. The risks associated with triple therapy in cirrhotics were perhaps best outlined by the prospective observational multi-center French study (ANRS-CUPIC) study which compared on-treatment response with TVR (*n* = 285) and BOC-based (*n* = 204) triple therapy in compensated genotype 1 cirrhotics who were partial responders or relapsers to prior dual therapy[8]. The week 16 on-treatment virologic response data showed that 67% of the TVR group and 58% of the BOC group had undetectable HCV viral loads. The high rate of SAEs in both groups (33%-45%) contrasted starkly to the 9%-14%

Table 2 Treatment of hepatitis C cirrhosis with triple therapy

Fibrosis stage	Drug	Prior PEG-IFN responsiveness (*n*)	Treatment response	Serious adverse events[1]	Ref.
Cirrhosis	Telaprevir	21	62% SVR	9%	[4]
Bridging fibrosis and cirrhosis	Boceprevir	76	47% SVR	12%	[5]
		Relapse (*n* = 119)	85% SVR		
		Partial response (*n* = 50)	42% SVR		
		Null response (*n* = 88)	24% SVR		
Bridging fibrosis and cirrhosis	Boceprevir	Relapse and partial response (*n* = 63)	56% SVR	12%	[7]
Child A	Telaprevir	285	67% (16 wk)	45%	[8]
	Boceprevir	204	58% (16 wk)	33%	

[1]Serious adverse event rate for entire study population, % of patients with undetectable hepatitis C virus RNA at 16 wk following drug initiation, an interim analysis. PEG-IFN: Pegylated-interferon; SVR: Sustained viral response rate.

SAE rate seen in the phase 3 trials that led to licensing of the protease inhibitors. Anemia was especially problematic as 46%-54% of patients required erythropoietin and 6%-16% required blood transfusions[8]. The availability of better and safer next generation direct acting antiviral therapy, such as sofosbuvir, has quickly caused providers to abandon first generation triple therapy with TVR and BOC as a treatment strategy.

NATURAL HISTORY OF HEPATITIS C AFTER LIVER TRANSPLANT

HCV infection recurs universally in LT recipients who are viremic at transplantation. Viral kinetic studies have shown that replication begins as early as the first postoperative week and typically peaks by the fourth postoperative month. Virus levels at one year post-LT are 10-20 fold greater than pre-transplant[9]. Histologic studies have shown accelerated fibrosis progression compared to immunocompetent patients infected with HCV. A retrospective cohort study of 183 liver transplant recipients with HCV looked at fibrosis progression based on protocol liver biopsies done over a 10 year period. Fibrosis progression was non-linear, increasing exponentially during the first three years post-LT. Having advanced fibrosis (> stage 2) 1 year post-LT led to a 15-fold increase in HCV-related graft loss[10]. Cirrhosis occurs in up to 20% of patients within 5 years of LT. The cumulative probability of decompensation 1 year after developing cirrhosis is 30%. Once decompensated cirrhosis occurs, the 1 year-survival rate is poor at 46%[11].

CLASSIFICATION OF RECURRENT HEPATITIS C

Standardized definitions of recurrent hepatitis C and its various forms were proposed by an international consensus conference and published in 2003[12]. Recurrent HCV infection is defined by the presence of HCV RNA in serum and/or liver. Acute recurrent HCV is often associated with elevated aminotransferases and typically occurs within 6 mo of LT, though it can occur any time post LT. Histologically, reinfection of the graft with HCV is char-

acterized by lobular hepatitis, focal hepatocyte necrosis, acidophil bodies, and macrovesicular steatosis. Chronic recurrent HCV disease develops as a result of acute recurrent HCV. Liver biopsy findings include chronic hepatitis with mixed portal, periportal, and lobular inflammation with variable degrees of portal and periportal fibrosis[12].

A more detailed set of criteria was proposed for fibrosing cholestatic hepatitis C (FCH). This includes (1) onset greater than 1 mo and usually < 6 mo after LT; (2) serum bilirubin greater than 6 mg/dL; (3) serum alkaline phosphatase and gamma-glutamyltransferase levels greater than 5 times the upper limit of normal; (4) characteristic histology with hepatocyte ballooning, a paucity of inflammation, and cholestasis; (5) very high HCV RNA levels; and (6) absence of biliary or vascular complications[12].

IMPACT OF DONOR AND RECIPIENT RISK FACTORS ON HCV RECURRENCE AFTER TRANSPLANT

Risks due to donor allograft

The challenge to mitigate the risk of HCV recurrence begins prior to transplant with the selection of the appropriate donor allograft when possible. Donor age influences the risk of recurrent HCV and graft survival. For HCV positive recipients, donor age over 40[13] (or 50[14]) years old was found to be an independent predictor of graft loss and patient death in two large retrospective reports of liver transplant recipients from the Scientific Registry of Transplant Recipients (SRTR) and United Network of Organ Sharing (UNOS) databases. Recent data also points to an association between the incidence of FCH and the use of allografts from older donors. Verna *et al*[15] reviewed 179 post-LT biopsies that had been initially categorized as demonstrating cholestatic hepatitis C and refined the classification of FCH to include only those patients meeting at least 3 of the following 4 pathologic criteria: (1) ductular reaction; (2) cholestasis; (3) hepatocyte ballooning with lobular disarray; and (4) periportal sinusoidal/pericellular fibrosis (Figure 1). With these more stringent standards, donor age (OR
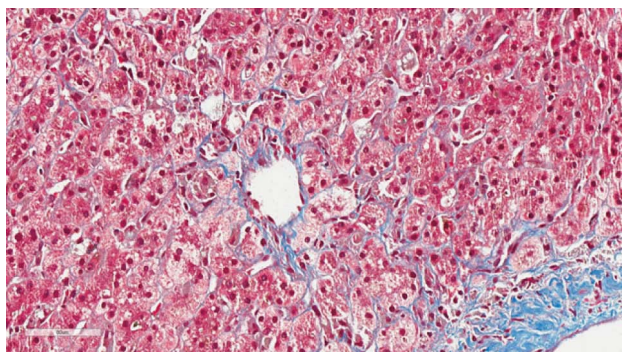
Figure 1 Histopathology of fibrosing cholestatic hepatitis C. Histopathology of fibrosing cholestatic hepatitis C demonstrating periportal sinusoidal and pericellular "chicken wire" fibrosis (trichrome, image magnification × 40) (Courtesy of Carl Jacobs, MD, Department of Pathology, Carolinas Medical Center, Charlotte, NC, United States).

= 1.37, 95%CI: 1.02-1.84, P = 0.04) and prior history of acute cellular rejection (OR = 4.19, 95%CI: 1.69-10.4, P = 0.002) were the strongest predictors of developing FCH on multivariate analysis. Thus, transplanting older allografts into HCV recipients leads to worse outcomes due to recurrent HCV both in the short and long-term.

Weighing the risk-benefit ratio of selecting an allograft from a donor of advanced age reflects the imbalance between supply and demand in liver transplantation today. Another consequence of this disparity is the increased reliance on potential donors with hepatic steatosis. Overall, recipients of allografts with over 30% macrovesicular steatosis are at increased risk of delayed graft function and primary graft non-function. However, the natural history of hepatitis C recurrence in recipients of fatty livers is less clear. Interestingly, macrovesicular steatosis at the time of transplant has been shown to be a transient and reversible phenomenon[16,17]. As such, the mechanistic link between allograft steatosis and HCV reinfection is ambiguous. In a prospective study comparing 56 HCV[+] and 60 HCV LT recipients[18], Burra and colleagues did not find a statistically significant difference between donor biopsy steatosis and patient or graft survival at 3 years. However, only 9.5% (11/116) of the donor biopsies in their study population had > 33% steatosis, limiting their ability to detect a statistical difference. In contrast, Briceño *et al*[19] found an inverse relationship between graft survival and donor liver steatosis in 120 HCV[+] LT recipients, of whom 48/120 (40%) had > 30% donor steatosis. One year following LT, 40% of patients who received a donor allograft with > 30% steatosis had histologic evidence of HCV recurrence with ≥ stage 2 fibrosis in comparison to only 17% in patients who received a graft with < 30% steatosis. Their conclusion that HCV recurrence is more aggressive in patients receiving allografts with moderate/severe steatosis is subject to selection bias, though, since their enrollment included only patients biopsied for elevated aminotransferases[20]. In summary, the presence of allograft steatosis > 30% can be problematic for any LT recipient, but particularly in the context of other extended donor criteria such as ad-

vanced donor age or cold ischemic time > 10 h. Whether steatosis in and of itself modifies the risk of HCV recurrence remains subject to interpretation and undoubtedly the topic of future investigation.

The influence of HCV recurrence has been studied in regard to split liver and living donor liver transplants as well. Two studies comparing recipients of either a deceased or living donor liver transplant did not find any difference in HCV recurrence or graft survival at 2[21] and 3[22] years. A study by Selzner *et al*[23] found that HCV[+] recipients of a living donor liver transplantation (LDLT) had less fibrosis progression at 24 mo than deceased donor HCV[+] transplant recipients. Not surprisingly, the average donor age of the LDLT recipients was younger than that deceased donor liver transplantations (DDLTs). Age > 45 years old was the only variable independently associated with fibrosis progression from their cohort of 46 LDLTs and 155 DDLTs. Since split liver transplant recipients receive younger organs on average, it is not surprising that no differences have been shown in the small studies[24,25] with these grafts, either. Interestingly, Yoshizawa *et al*[26] reported two cases in which identical twins underwent LDLT from their respective siblings. In both cases, HCV recurrence occurred shortly after transplant even in the absence of immunosuppression. Fortunately, both patients responded well to antiviral therapy.

Peri-operative factors to take into consideration include ischemia-reperfusion injury (IRI) and ischemia time. Several factors contribute to IRI, most notably donor status (cardiac *vs* brain death); and warm and cold ischemic time. A small, but well-controlled study by Watt *et al*[27] demonstrated increased mortality at 3 years (59% *vs* 82%, 59% *vs* 88%, P = 0.0055) in HCV[+] recipients with preservation injury (PI) compared to non-HCV recipients with PI and HCV[+] recipients without PI, who were matched for gender, age, and immunosuppression. In contrast, time to histologic HCV recurrence did not correlate with the severity of IRI as defined biochemically by peak alanine aminotransfersase levels and on liver biopsy in a large retrospective cohort of HCV[+] transplant recipients[28]. The impact of IRI on the risk of HCV recurrence remains unclear due to potential confounding from perioperative and donor factors. Taner *et al*[29] attempted to address confounding by exclusively comparing HCV[+] recipients of a donation after cardiac death (DCD) allograft to HCV recipients of a DCD organ and HCV[+] recipients of a brain dead donor graft. In this large, single center experience no difference in patient or graft survival was found up to five years after transplant.

Lastly, HCV[+] donors constitute a subset of extended criteria donor allografts that may be considered given the organ shortage in liver transplantation. Marroquin *et al*[30] studied UNOS data from 1994-1997 and identified 96 HCV[+] allografts transplanted into HCV[+] recipients, who were more likely to have been transplanted due to underlying hepatocellular carcinoma (8.3%) than those patients receiving an HCV allograft (3.1%). Patients who received the HCV[+] allografts had improved survival at 24

mo compared HCV allograft recipients (90% *vs* 77%, *P* = 0.01). A retrospective case control study of 39 HCV[+] allografts transplanted over a 16-year period also showed no difference in overall survival, though fibrosis progression was more advanced in the HCV[+] allograft recipients, and particularly those patients who received an HCV[+] organ from a donor over age 50[31]. The synergism in risk encountered by transplanting an HCV[+] organ from a donor > 45 years old was recently reaffirmed by the CRUSH-C investigators[32]. Nonetheless, survival data up to 5 years following OLT with HCV[+] donors[33] confirms the utility of these organs for genotype 1-positive recipients who have not achieved a sustained virological response to anti-HCV therapy before transplantation. With the promise of direct acting antivirals for HCV now in view, transplant centers may continue to make use of these organs for appropriate patients.

Viral factors

HCV genotype affects recurrent hepatitis C with fibrosis progression from stage 1 to 2 more common in HCV genotype 1 transplant recipients compared to non-1 genotypes (HR = 2.739, 95%CI: 1.047-7.143, *P* = 0.04)[29]. Pre and post-transplant viral loads appear to influence the risk of HCV recurrence as well. A pre-transplant viral load > 1×10^6 vEq/mL was associated with increased mortality at 5-years post LT from an early study[34]. However, when pre-transplant HCV RNA level was incorporated into a model by the same investigators to predict outcomes in HCV[+] transplant recipients, no difference in survival at 10 years was seen when compared to uninfected transplant recipients[35]. Post-transplant HCV RNA ≥ 1×10^9 copies/mL at 4 mo following LT was associated with worse necroinflammatory activity as assessed by hepatitis activity index on protocol biopsies at 1 and 3 years following LT[36]. Hanouneh *et al*[37] also reported an independent association (HR = 1.1, *P* = 0.004) between fibrosis progression and HCV RNA level at 4 mo following transplant.

Cytomegalovirus (CMV) co-infection has consistently been shown to impact the risk of HCV recurrence. CMV infection, defined as viremia requiring antiviral therapy, resulted in graft failure in 52% *vs* 19.1% (*P* = 0.002) of 93 consecutive HCV[+] transplant recipients[38]. Fibrosis (stage ≥ 2) on protocol liver biopsies at month 4 was significantly higher (45% *vs* 16.4%, *P* = 0.01) in the co-infected patients compared to the CMV-group. CMV co-infection was associated with fibrosis progression by univariate analysis in a study of HCV[+] transplant recipients with and without metabolic syndrome[37]. In the aforementioned study on the risk of DCD donation in HCV[+] patients, CMV infection post-transplant was a significant factor for graft loss (HR = 3.367, 95%CI: 1.493-7.593, *P* = 0.003)[29]. CMV viremia may alter the host immunological profile independent of its effect on HCV replication[39].

Recent prospective data from a US consortium of human immunodeficiency virus (HIV)/HCV investigators establishes HIV co-infection as a significant risk for graft failure[40]. Patient survival at 3 years post-LT was 60% in 89 HCV/HIV-co-infected patients compared to 75% in 235 HCV mono-infected patients (*P* < 0.001). However, after excluding 25 (28%) co-infected patients who met at least one of the following criteria: (1) a body mass index < 21 kg/m²; (2) a HCV[+] donor allograft; or (3) a combined liver kidney transplant; patient survival was not statistically different in comparison to all the United States transplant recipients ≥ 65 years old or liver transplant recipients transplanted for other indications. Acute rejection occurred more frequently in co-infected patients (39% *vs* 24%, HR = 2.1, *P* = 0.01), but HCV disease severity assessed on biopsy was not statistically different between the two groups. This finding is in contrast to the study by Duclos-Vallée *et al*[41] where time to fibrosis progression (stage ≥ 2) was significantly shorter in the co-infected population. The failure to detect a difference in fibrosis progression in the United States cohort is almost certainly a limitation in power (only 62% of HIV/HCV patients had liver biopsies and multiple deaths occurred early from sepsis and multiorgan failure), though it may also reflect different centers' thresholds to begin antiviral therapy. In summary, HIV infection likely impacts HCV fibrosis progression in co-infected transplant recipients. Careful patient selection is essential in order to achieve good outcomes.

Host factors

A number of studies identify sex and race as modifying the risk of HCV recurrence following transplant. The CRUSH-C investigators found that HCV[+] women were at increased risk for bridging fibrosis or cirrhosis after transplant in comparison to their HCV[+] male counterparts[42]. After multivariate analysis, female sex was found to be an independent predictor of advanced fibrosis (HR = 1.31, 95%CI: 1.02-1.70, *P* = 0.04) and mortality (HR = 1.30, 95%CI: 1.01-1.67, *P* = 0.04). African American patients transplanted for HCV who receive an allograft from a racially matched donor have been shown to have excellent outcomes following transplant. Conversely, African American HCV[+] recipients of a racially mismatched allograft are at increased risk of graft failure and death. Pang *et al*[43] reviewed UNOS data from 1998 through 2007 and found a 5-year survival rate of 45% for racially mismatched pairs compared to 59% for a racially matched pairs. The survival rates for African American matched donor and recipient pairs was on par with HCV[+] Caucasian transplant recipients. The risk associated with a mismatched donor was restricted to HCV[+] African American recipients and not HCV negative African American transplant recipients. Two studies recently published by Saxena *et al*[44] and Layden *et al*[45] reinforced the survival data by finding that racial mismatch is a significant independent predictor of advanced fibrosis. While the exact interaction between HCV, the host immune system, and the donor allograft genetic profile isn't clear, the overwhelming data supports that racial mismatch is associated with poor outcomes in HCV[+] African Ameri-

Factor	Evidence
Table 3 Risk factors studied for association with more severe hepatitis C virus recurrence	
Donor	
Age > 40 yr	↑↑↑
Living donor	↔
Split liver	↔
DCD	↔
HCV+	↔
Macrovesicular steatosis > 30%	↑↓
IRI	↑↓
IL28B "CC" genotype	↑↓
Virus	
HCV genotype 1	↑
High pre-transplant HCV RNA	↑
HCV RNA 4 mo post LT ≥ 1 × 10^9 mEq/mL	↑↑
CMV viremia	↑↑↑
HIV coinfection	↑↑
Recipient	
Female sex	↑↑
African American D/R mismatch	↑↑
African American D/R match	↔
Metabolic syndrome[1]	↑
IL28B non-"CC" genotype	↑
Immunosuppression	
Pulsed corticosteroids for American College of Rheumatology	↑↑↑
Tacrolimus (*vs* CsA)	↑↓
Sirolimus	↑
Thymoglobulin	↔
Basiliximab	↔
OKT3	↑

[1]Metabolic syndrome defined by ATP-III criteria 1 yr post liver transplantation (LT)[37], homeostasis model assessment-estimated insulin resistance > 2.5 4 mo post LT[46], or hepatic steatosis ≥ 5% on an index liver allograft biopsy 1 yr post-LT[47]. ↑: Evidence of increased risk; ↔: Evidence of no increased risk; ↑↓: Indeterminate risk; DCD: Donation after cardiac death; HCV: Hepatitis C virus; CMV: Cytomegalovirus; HIV: Human immunodeficiency virus; CsA: Cyclosporin A; OKT3: Muromonab-CD3; IRI: Ischemia reperfusion injury.

can transplant recipients due to recurrent HCV disease.

Metabolic syndrome (MS) following LT is associated with worse outcomes after LT and may be an important modifiable risk factor. Hanouneh *et al*[37] reported an independent association between metabolic syndrome and fibrosis progression in HCV+ transplant recipients 1-year following OLT. Similarly, Veldt *et al*[46] calculated the homeostasis model assessment of insulin resistance (HOMA-IR) in 160 HCV+ patients 4 mo following transplant and found that insulin resistance as defined by a HOMA-IR > 2.5 was independently associated with advanced fibrosis (HR = 2.07, 95%CI: 1.10-3.91, *P* = 0.024). The above two studies suggest a link between MS and fibrosis progression in HCV+ transplant recipients. Whether the influence of MS on fibrosis progression is specific to HCV+ recipients or all transplants remains a question and further studies are needed. Lastly, HCV+ transplant recipients with hepatic steatosis (≥ 5%) on an index liver allograft biopsy 1 year post-LT may be at increased risk of fibrosis progression[47]. The cumulative rate of significant fibrosis (Ludwig-Batts F2-F4) after a median follow-up of 2 years after an index biopsy was 49% compared to 24% for HCV+ recipients without steatosis on their index

biopsy. Overall, MS is an important consideration following liver transplant for any cause, particularly in view of the side effects of lifelong immunosuppression. A comprehensive assessment for MS should be integrated into post-transplant care, especially in the HCV+ and nonalcoholic fatty liver disease population (Table 3).

IL28B genotype

Genetic variation upstream of the IL28B gene was originally found in GWAS to be associated with response to interferon therapy as well as spontaneous clearance after acute HCV infection, with the presence of the "CC" genotype for rs12979860 predicting both of the above favorable outcomes in the non-transplant setting. The impact of donor and recipient *IL28B* genotype (for rs12979860) on HCV recurrence and outcomes following liver transplant has recently been the topic of a number of studies[48,49]. Duarte-Rojo *et al*[50] found that donor-CC genotype allograft recipients had a significantly higher average ALT, viral load, and rate of fibrosis ≥ stage 2 at 1 year compared to non-CC donor graft recipients. On the other hand, recipient "CC" genotype was associated with the exact opposite result with rates of fibrosis ≥ stage 2 at 1 year of only 19% compared to 38% for non-CC recipient genotype (*P* = 0.012). The combination of both donor and recipient "CC" genotype was associated with a 90% sustained virological response to antiviral therapy. Interestingly, Duarte-Rojo found that donor-CC genotype was independently associated with adverse outcomes - defined as cirrhosis, liver-related death, or re-transplantation. This finding was not a result of increased rates of acute cellular rejection, however. In contrast, a survival analysis performed recently by Allam *et al*[51] did not demonstrate the same impact on donor *IL28B* genotype, perhaps due to grouping of genotypes "CC" and "CT" together rather than comparing "CC" with non-CC genotypes. While it is safe to conclude that the unfavorable "T" allele in recipients is associated with a worse response to antiviral therapy as in the pre-transplant setting, a consensus on the influence of donor and recipient IL28B on outcomes following LT in HCV+ patients has yet to emerge. A comprehensive review of *IL28B* genotype in transplantation for HCV is beyond the scope of this article, but the topic of an excellent recent review[52]. Hopefully, the promise of direct acting antiviral therapy will reduce the impact of *IL28B* genotype.

Immunosuppression: Corticosteroids

Immunosuppression represents arguably the most critical factor to address with respect to HCV recurrence following transplantation. A balance exists between maintaining appropriate immunosuppression and preventing aggressive HCV recurrence. HCV+ recipients who receive high dose steroid treatment for acute cellular rejection are at risk for developing FCH. Donor age (OR = 1.37, *P* = 0.04) and previous rejection defined as Banff grade ≥ 5 (OR = 4.19, *P* = 0.002) were found to be the two most important predictors of developing FCH[15]. The associa-

tion between steroid bolus therapy and early graft loss as well as death has been reported by others[54,53]. Data on the impact of maintenance steroids following liver transplantation for HCV is less clear. The decision and timing to stop steroids varies across transplant centers[54]. Nonetheless, a meta-analysis by Segev *et al*[55] reported a reduced risk of HCV recurrence for steroid free regimens (RR = 0.90, *P* = 0.03), even though no individual trial met statistical significance. A recent study by Takada *et al*[56] evaluated the impact of steroid avoidance on HCV recurrence following LDLT. Seventy-five patients were randomized to immunosuppression with either tacrolimus (TAC) plus a corticosteroid or TAC with mycophenolate mofetil (MMF). HCV recurrence rates defined by a METAVIR fibrosis score ≥ 1 were not statistically different at either 1 or 3 years following transplant.

Calcineurin inhibitors

Cyclosporine (CsA) has been shown to inhibit hepatitis C viral replication *in vitro*, however, evidence supporting a benefit over tacrolimus with regard to HCV recurrence and fibrosis progression following transplant is inconsistent. The LIS2T trial was a prospective, open-label, randomized trial comparing CsA to tacrolimus[57]. In the HCV+ transplant recipients, death and graft loss were higher with tacrolimus compared to CsA (16% *vs* 6%, *P* ≤ 0.03) at 12 mo. However, it's not clear that any of those deaths occurred as a result of recurrent or fibrosing cholestatic hepatitis C. Moreover, there was no difference in fibrosis stage between the CsA and tacrolimus groups. A meta-analysis by Berenguer *et al*[58] that included 5 trials, totaling 366 patients failed to detect a difference between CsA *vs* tacrolimus-based regimens. A subsequent prospective study comparing CsA and tacrolimus by Berenguer *et al*[59] involved 253 patients transplanted for HCV between 2001 and 2007. Severe recurrent disease defined as bridging fibrosis, cirrhosis, FCH, graft failure, and death occurred with the same frequency in both groups (CsA: 27% *vs* Tacrolimus: 26%, *P* = 0.68)

The decision to incorporate a CsA-based immunosuppression strategy in HCV+ patients after transplant typically revolves around beginning antiviral therapy for recurrent disease (see discussion below). Inhibiting HCV replication with CsA *in vivo* might conceivably augment the response to PEG-IFN and RBV. A study by Firpi *et al*[60] supported this claim with improved SVR rates with CsA *vs* tacrolimus (46% *vs* 27%, *P* = 0.03). However, a smaller controlled trial of mostly genotype 1 patients randomized to either CsA or tacrolimus did not find a difference in SVR rates (39% *vs* 35%, *P* = 0.8)[61].

mTOR inhibitors

Sirolimus may be prescribed after liver transplantation in patients intolerant to calcineurin inhibitors or for hepatocellular carcinoma or as primary immunosuppression. SRTR data from 26414 liver transplants (12589 for HCV) was analyzed to address risk factors for patient and graft survival[62]. 6.5% (795/12269) of HCV+ trans-

plant recipients were prescribed sirolimus at the time of discharge from LT, and 3.5% of these patients remained on the drug 1 year after transplant. Sirolimus was found on multivariate analysis to be associated with increased mortality within three years of liver transplant in HCV+ recipients (HR = 1.26, 95%CI: 1.08-1.48, *P* = 0.0044), but not in non-HCV patients. On the other hand, all patients (HCV+ or HCV) on tacrolimus-based regimens had improved overall survival. The authors performed a propensity analysis to account for the fact that patients who received sirolimus were more likely to have had HCC and also had significantly higher creatinine and MELD score prior to transplant. Sirolimus at baseline was still an independent risk factor for increased mortality at 3 years (HR = 1.29, 95%CI: 1.08-1.55, *P* = 0.0053). While the SRTR database does not capture biopsy data to determine whether the increased mortality in the sirolimus group was a result of HCV recurrence, the mortality data certainly warrants pause and further investigation into the mechanism which may underlie the association. The larger studies with everolimus in LT from Toronto[63] and Italy[64] have been powered to determine efficacy, safety, and renal protective benefits and not the impact on HCV recurrence.

MMF, T-cell depleting therapies, and IL-2 receptor inhibition

The addition of MMF to immunosuppression regimens in the mid to late 1990s has had a positive impact on long-term outcomes following LT for all causes including hepatitis C[65]. The data for thymoglobulin induction suggests that it is safe to use in HCV+ patients and that it may provide a benefit in terms of slowing fibrosis progression[66]. This benefit may derive from lower rates of acute cellular rejection. Interestingly, a study comparing outcomes with routine induction with either thymoglobulin or basiliximab after living donor liver transplant demonstrated more frequent HCV recurrence requiring antiviral therapy in patients who received rabbit thymoglobulin[67]. Data demonstrate the safety profile of basiliximab induction therapy in HCV+ transplant recipients. A randomized trial comparing basiliximab with or without steroids indeed found that the steroid free group had less fibrosis at 6 mo, 1 year, and 2 years following transplant[68]. With the exception of OKT3, which has historically been associated with poor outcomes when used in HCV+ transplant recipients[69], the overall evidence suggests an acceptable safety profile with the use of thymoglobulin or basiliximab.

ROLE OF PROTOCOL LIVER BIOPSIES

Transplant centers may perform annual protocol liver biopsies on HCV transplant recipients to assess disease progression. However, there is a lack of uniformity regarding their use. A study from Spain evaluated protocol liver biopsies from 245 patients between 1991 and 1997. HCV infection +/- alcohol was the cause of cirrhosis in

Table 4 Liver biopsy findings in recurrent hepatitis C and acute cellular rejection

Favors hepatitis C	Favors rejection
Apoptotic (Councilman) bodies	Central venulitis
Lymphoid aggregates	Perivenular necrosis
Kupffer cell hypertrophy	Inflammatory bile duct damage
Mononuclear portal inflammation	Biliary epithelial senescence changes
Ballooning degeneration	

125 patients. Histologic evidence of recurrent hepatitis C was present in 66% of patients at 1 year and 80% at 3 years post-LT. The cumulative probability of developing stage 3 or 4 fibrosis was 41% at 5 years post-LT[70]. Based on these results, the authors concluded that the high prevalence of abnormal histology and the rate of fibrosis progression justify the use of protocol biopsies.

A cohort of 264 HCV-infected liver transplant recipients who underwent protocol liver biopsies showed that the 12-mo biopsy had the best ability to stratify fibrosis progression. Twenty one percent of patients with stage 2-3/6 fibrosis at month 12 progressed to cirrhosis (stage 5-6) within 5 years. The degree of inflammation also correlated with fibrosis progression[71]. These studies support the role of protocol liver biopsies and suggest that rapid fibrosis progressors can be identified within a year of transplant.

LIVER BIOPSY INTERPRETATION AFTER LIVER TRANSPLANTATION

One of the most challenging diagnostic dilemmas for the hematopathologist and transplant team is distinguishing recurrent hepatitis C from acute cellular rejection on liver biopsy. The treatments are diametrically opposed; treatment of rejection requires more intensive immunosuppression which may exacerbate recurrent hepatitis C and treatment of hepatitis C with interferon based therapy may provoke an immune mediated injury or plasma cell hepatitis.

An understanding of the timing of reinfection and features on liver biopsy can help distinguish recurrent hepatitis C from rejection. Immediately after liver transplant hepatitis C virus infects the liver allograft. Within the first several weeks after liver transplantation serum HCV RNA levels are approximately 1-log higher compared to non-transplant HCV patients. Elevations in aminotransferases and an acute hepatitis with a lobular lymphocytic infiltrate may be seen two to six months after transplant. Thus, monitoring HCV viral load within the first 3 mo after transplant may be helpful because high viral load may support recurrent hepatitis C as a cause for elevated liver tests.

Although recurrent hepatitis C and acute cellular rejection may have similar pathologic findings on liver biopsy several features on biopsy may be helpful in distinguishing the two diagnoses (Table 4). The histopathologic variants of hepatitis C that have been described include usual or conventional hepatitis C, fibrosing cholestatic hepatitis C, plasma-cell rich hepatitis C, and HCV overlapping with acute and chronic rejection. The plasma cell rich hepatitis is seen in patients on interferon based antiviral therapy who typically are on low levels of immunosuppression and have low or undetectable serum HCV RNA. Plasma cells are not a feature of acute cellular rejection, although they may be seen in antibody mediated rejection.

HCV evolution in liver allografts in the acute phase of reinfection during the first 1-3 mo after liver transplant shows lobular disarray, Kupffer cell hypertrophy, hepatocyte apoptosis, macrovesicular steatosis, mild sinusoid lymphocytosis and portal inflammation[72]. Damage of bile ducts by infiltrating lymphocytes is usual mild, in contrast to acute cellular rejection where biliary epithelium damage can be severe. This constellation of findings can be useful in distinguishing recurrent hepatitis C from acute cellular rejection (Table 4).

Molecular and immunologic diagnostics have been used to distinguish hepatitis C from rejection or to identify recipients with aggressive hepatitis C[73-76]. A cirrhosis risk score was developed from a 7-gene signature that accurately identified liver transplant recipients who developed advanced fibrosis on liver biopsy after liver transplantation[73]. A prospective study of an immune functional assay found that the immune response was significantly higher in recipients with features of acute cellular rejection on liver biopsy compared to recipients with features of recurrent hepatitis C ($P < 0.001$)[74]. Hepatitis C recurrence has been associated with genes associated with cytotoxic T cells profile and acute cellular rejection was associated with an inflammatory response gene profile[75]. Increased expression of miRNA-146a, miRNA-19a, miRNA-20a, and miRNA-let7e was seen in hepatitis C recipients with slow fibrosis progression[76]. Molecular profiling is not widespread and needs further validation for distinguishing rejection from hepatitis C recurrence or for identifying hepatitis C recipients at greatest risk for progressing to cirrhosis.

TREATMENT OF RECURRENT HCV AFTER TRANSPLANT

Cirrhosis develops in approximately 20% of HCV+ transplant recipients within 5 years of LT[77,78]. The 5-year survival following a liver transplant for HCV is significantly worse than for non-HCV related disease (69.9% *vs* 76.6%, $P < 0.0001$)[79]. Furthermore, only 25% of patients treated with standard dual therapy following transplant (PEG-IFN and RBV) achieve an SVR. Thus, the impact of direct acting antiviral therapy (DAA) for HCV cirrhotic patients awaiting transplant and for patients with recurrent disease requiring treatment, is potentially life prolonging - if not life altering.

Preventing allograft reinfection by treating HCV+ patients awaiting transplant will continue to be a key objective. As discussed above, clinical trials with dual

therapy (PEG-IFN and RBV) in HCV[+] patients awaiting transplant have had disappointing rates of SVR following LT (Table 1). Triple therapy (PEG-IFN, RBV, and either TVR or BOC), even in compensated cirrhotics, is poorly tolerated and risks serious adverse outcomes in Child's A patients[8]. Interferon free trials with DAAs in patients awaiting transplant is an exciting area of research. One such trial recently presented in abstract form is (NCT01559844), an open-label study with the NS5B polymerase inhibitor, sofosbuvir, and RBV, which was designed specifically to determine the SVR rate 12 wk following LT in patients with HCV and HCC awaiting transplant[80]. Of the 26 patients who received sofosbuvir and RBV prior to transplant and who had HCV RNA levels < 25 IU/mL just prior to LT, 18 (69%) attained an SVR 12-wk following LT. Based upon this compelling data, sofosbuvir gained FDA approval with an additional indication for treatment in the pre-transplant setting for up to 48 wk in combination with RBV in chronic hepatitis C patients with HCC awaiting LT. This strategy promises to have an immediate and profound impact on HCV[+] transplant recipients. Finally, antibody therapy directed against the HCV envelope protein is another tactic under investigation to prevent allograft reinfection which may yet prove efficacious particularly in combination with DAAs[81].

No data supports pre-emptive antiviral therapy in the first six months after LT for HCV[82]. Nevertheless, this approach will likely be reevaluated when safer oral therapies are available. Early post-transplant therapy is reserved for patients with FCH, who are at risk for rapid graft failure without treatment. Unfortunately, PEG-IFN and RBV are rarely successful for FCH, and the addition of boceprevir or telaprevir has not afforded dramatically improved response rates, either. Moreover, triple therapy is even harder for these typically decompensated patients to tolerate. The pharmaceutical industry, recognizing the high mortality associated with a diagnosis of FCH has made some of their DAAs available to physicians on a compassionate use basis with some excellent reported outcomes[83,84].

Apart from FCH, antiviral therapy for recurrent HCV after liver transplant is typically reserved for those patients with at least stage 2 fibrosis and/or moderate to severe necroinflammatory activity on liver biopsy. A meta-analysis by Wang *et al*[85] evaluated the efficacy of PEG-IFN and RBV after transplant and found a pooled SVR rate of 27% and a pooled discontinuation rate of 26%. The decision to begin dual therapy must also weigh the risk of immune-mediated graft dysfunction developing from exposure to PEG-IFN[86]. Historically, maintenance therapy with long-term, low-dose PEG-IFN, with or without RBV, has been used with a hope of delaying progression of fibrosis in recurrent HCV patients who do not attain an SVR with treatment after transplant, but do achieve a reduction in viral load and improvement in LFTs with treatment. However, no clinical trial has demonstrated a benefit of this approach.

Given the unsatisfactory SVR rates for standard therapy, it is not surprising that a flurry of off-label use of TVR and BOC began when the first generation protease inhibitors became available for genotype 1 patients. Triple therapy after transplant is particularly challenging since TVR and BOC inhibit cytochrome P450 3A4, which is responsible for the metabolism of CsA and tacrolimus. CsA levels increased approximately 4.6-fold following co-administration with TVR in healthy controls subjects while the corresponding number with tacrolimus was 70-fold[87]. An analysis of pharmacokinetic data with TVR 1125 mg BID combined with PEG-IFN/RBV in liver transplant recipients requiring HCV therapy was recently presented[88]. Among the 19 subjects, 16 were maintained on tacrolimus. With co-administration of TVR, the average dose of tacrolimus was 0.5 mg given at an average interval of every 168 h. (range = 96-607 h). Transplant centers are carefully utilizing triple therapy, often, but not exclusively, after converting patients from tacrolimus to CsA. Pungpapong *et al*[89] recently reported the Mayo experience of 66 patients who received triple therapy. TVR was given for 12 wk in combination with PEG-IFN (starting dose 135 µg weekly) and RBV, followed by PEG-IFN and RBV for 36 wk. The CsA dose was reduced by 75%-100%. Sixty-seven percent (14/21) of TVR-treated patients had an undetectable HCV RNA at week 24 in this preliminary analysis. Forty-five percent (10/22) of the BOC-treated patients had undetectable HCV RNA at week 24. Dose reductions of PEG-IFN were required for leukopenia in 75% of patients and RBV dose reductions occurred in all but 4 of the 66 study patients. Recent results from the CRUSH-C consortium confirm similar efficacy with triple therapy[90] (Figure 2). Ninety-six percent of the 112 patients studied had a lead-in period of dual therapy prior to beginning TVR (88%) or BOC (12%). The more frequent calcineurin inhibitor used was CsA (61%) with an average dose reduction of 75%. The median time from liver transplant for the population was 3.7 years and 84% had ≥ stage 2 fibrosis. Forty-three patients had follow-up of sufficient duration to measure an HCV viral load 4 wk (SVR4) after completing 48 wk. The SVR4 in this group was 65%. Ideally, the natural history of hepatitis C infection after transplant will change significantly with the availability of DAA, but these rates are encouraging for those patients who have not had the luxury of waiting for the newer agents.

RE-TRANSPLANT FOR HEPATITIS C

While re-transplantation for primary non-function or hepatic artery thrombosis is generally accepted, re-transplant for allograft failure due to recurrent HCV is a more contentious issue. A United States study group comprising 11 transplant centers compared survival after re-transplantation in patients with recurrent HCV and those re-transplanted for other indications. The overall 1-year and 3-year survival rates were lower, but not significantly
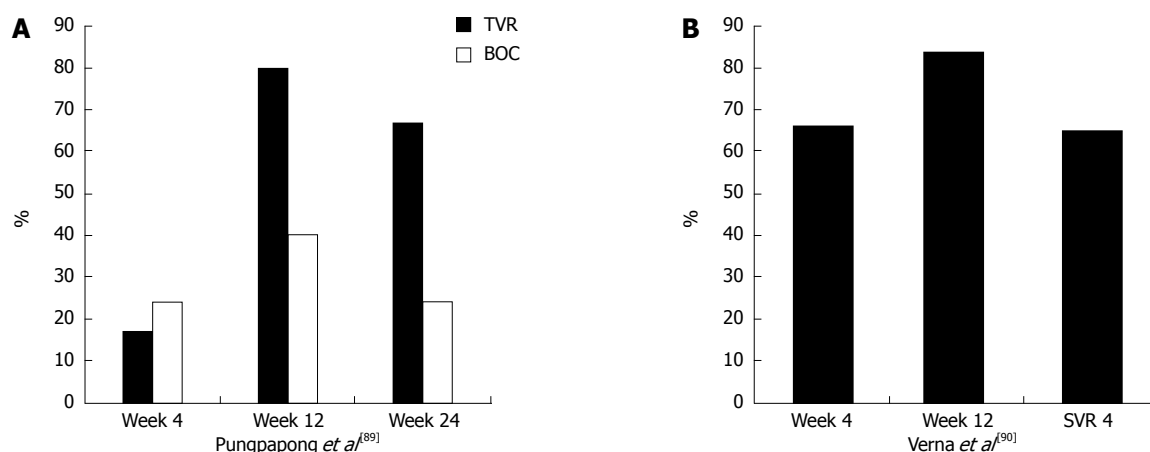
Figure 2 Percent of patients with hepatitis C virus RNA. A: Percent of patients with undetectable hepatitis C virus (HCV) RNA treated with transplant viral clearance (TVR) or boceprevir (BOC) plus pegylated-interferon/ribavirin post-liver transplantation[35]; B: Percent of patients on triple therapy (88%, TVR and 12%, BOC) with HCV RNA below level of detection. Sustained viral response rate (SVR4) refers to percent of patients with undetectable HCV RNA 4 wk following completion of 48 wk of treatment[36].

different, for patients re-transplanted for recurrent HCV (1 year, 69%, 3 year, 49%) compared to those re-transplanted for other causes (1 year, 73%, 3 year, 55%, *P* = 0.74). None of the 4 patients re-transplanted FCH were survived 1 year after re-transplant. Most notably, there seemed to be little enthusiasm for evaluating patients with HCV for re-transplant. Thirty percent of patients with allograft failure from recurrent HCV were not considered for re-transplant and only half of those evaluated for re-transplant were listed for transplant. The most common problems precluding re-transplant were recurrent HCV within 6 mo (22%), FCH (19%), and renal insufficiency (9%)[91].

There have been multiple studies evaluating risk factors of mortality following re-transplantation for HCV. Ghabril *et al*[92] evaluated 1034 HCV-infected patients and 1249 non-HCV-infected patients who underwent re-transplantation. Based on multivariate analysis, the independent predictors of mortality were recipient age, MELD score > 25, re-transplant during the first year after LT, donor age > 60, and a warm ischemia time of ≥ 75 min. Predictive models have been evaluated to select re-transplant candidates with the best potential outcomes. One such score was devised by focusing on HCV-infected patients from a large registry population. Variables included donor age, recipient age, creatinine, albumin, INR at the second transplant, and the interval between transplants. However, the receiver operating characteristic area under curve was a disappointing 0.643 at 3 years[93]. Though some of the above-mentioned risk factors are modifiable, performing re-transplants in patients with lower MELD scores using high quality donors may not be feasible given the donor shortage.

CONCLUSION

For more than a decade clinicians managing patients with hepatitis C awaiting liver transplant or who have had a liver transplant have been challenged in treating these patients. Antiviral therapy has been associated with substantial side effects with only modest efficacy. In addition, some liver transplant recipients develop rapid fibrosis progression while others coexist with the virus for years seemingly without any significant problems. Few modifiable factors have been identified to distinguish the two groups, although molecular markers hold promise as a predictive tool for fibrosis progression. The development of potent direct acting antiviral agents will hopefully obviate the need for interferon, and in the long-term provide a panacea that fundamentally changes the outcome for patients infected with this virus.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Changes in nutritional status after liver transplantation

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Abstract

Chronic liver disease has an important effect on nutritional status, and malnourishment is almost universally present in patients with end-stage liver disease who undergo liver transplantation. During recent decades, a trend has been reported that shows an increase in number of patients with end-stage liver disease and obesity in developed countries. The importance of carefully assessing the nutritional status during the work-up of patients who are candidates for liver replacement is widely recognised. Cirrhotic patients with depleted lean body mass (sarcopenia) and fat deposits have an increased surgical risk; malnutrition may further impact morbidity, mortality and costs in the post-transplantation setting. After transplantation and liver function is restored, many metabolic alterations are corrected, dietary intake is progressively normalised, and lifestyle changes may improve physical activity. Few studies have examined the modifications in body composition that occur in liver recipients. During the first 12 mo, the fat mass progressively increases in those patients who had previously depleted body mass, and the muscle mass recovery is subtle and non-significant by the end of the first year. In some patients, unregulated weight gain may lead to obesity and may promote metabolic disorders in the long term. Careful monitoring of nutritional changes will help identify the patients who are at risk for malnutrition or over-weight after liver transplantation. Physical and nutritional interventions must be investigated to evaluate their potential beneficial effect on body composition and muscle function after liver transplantation.

Core tip: Malnutrition, evidenced by muscle and fat depletion, represents a negative prognostic factor for morbidity and mortality in cirrhotic patients. This factor applies when liver transplantation is indicated. Nutritional depletion, as shown in the general population undergoing major surgery, may influence the outcome and global resource utilisation of liver transplantation. Recently, attention has focused on changes in nutritional status after liver transplantation. While fat mass is easily regained, muscle wasting, when present, is difficult to revert during the first year. The benefits derived from interventional programmes, such as exercise and dietary counselling, must be carefully evaluated in these types of patients.

INTRODUCTION

Liver transplantation has significantly changed the prognosis of end-stage liver disease. Improved immunosuppressive regimens and surgical techniques have progres-

sively modified the outcome of these patients, and the survival rate after liver transplantation is presently 82%, 71% and 61% at one, five and ten years, respectively[1].

From 2002, the introduction of the Model for End stage Liver Disease (MELD) for prioritising patients in need of new livers has led to a significant reduction in mortality for patients on the waiting list[2,3]. The utilisation of the MELD score, which is based on the serum concentrations of bilirubin and creatinine and the INR value, favours the transplantation of the sickest patients, in whom complications from cirrhosis are likely to be more severe and life expectancy is likely to be shorter.

Alterations in nutritional status are expected to be frequent in patients with advanced chronic liver disease both of alcoholic and viral origin[4-7]. These alterations, which may be recognised by more sophisticated methods, even in the early phases of liver cirrhosis[8,9], are in fact accelerated by the advanced stages of the disease. Depletions in muscle compartment and fat mass have been demonstrated to contribute to malnutrition in cirrhotic patients. Muscle wasting, which is accompanied by reduced muscle function (a condition defined as sarcopenia)[10], is likely the most relevant feature in these patients[11,12]. The prevalence of muscle wasting in liver cirrhosis has been reported to range from 10% to 70% according to gender and the severity of liver insufficiency (Table 1)[5,13-21]. A higher proportion of muscle wasting is generally observed in males, as muscle mass is physiologically lower in women; therefore, sarcopenia is less evident in females. Although less apparent, muscle wasting may be present in obese patients, which is identified as "sarcopenic obesity"[22,23] and underscores the importance of considering muscle depletion in these types of patients. This observation is relevant because the number of obese patients, with end stage liver disease due to Non Alcoholic Steato Hepatitis, is increasing among those who await liver transplantation[24,25]. A reduction in skeletal muscle mass in cirrhotic patients has been suggested to be an independent predictor of survival, quality of life, outcome and response to stress and surgery[11,26]. The negative predictive role of malnutrition in the outcome from major surgery confirms and extends the observation of the Michigan study[27]. Although many reports have recognised that malnutrition impacts liver transplantation outcomes, it is generally agreed that liver transplant should not be denied even in highly malnourished cirrhotic patients[28].

By restoring liver function, liver transplantation is expected to ameliorate the patient's nutritional status. In fact, many metabolic alterations that are involved in causing malnutrition in cirrhotic patients depend on the liver's inability to regulate energy metabolism and to maintain an adequate protein synthesis. Carbohydrate metabolism is impaired in cirrhosis because of the decreased liver glycogen deposits; transitioning to the fast state is, therefore, rapid in these patients, who need to activate gluconeogenesis to maintain adequate hepatic glucose production[29]. Insulin resistance is also a characteristic in these patients; the low hepatic insulin clearance contributes to this alteration[30]. Lipid turnover is activated due to an enhanced lipolysis, which allows an adequate availability of glycerol for gluconeogenesis and of free fatty acids as alternative energy sources[31]. Protein synthesis is impaired, reducing albumin and other transport proteins (such as lipoproteins). Furthermore, muscle protein catabolism is increased and provides alanine as a substrate for gluconeogenesis[32]. Reduced dietary intake caused by multiple factors (Table 2) is also recognised to contribute to malnutrition in cirrhotic patients. Inadequate food consumption associated with some degree of nutrient malabsorption is responsible for the vitamin and trace element deficiencies, which have been documented in end-stage liver disease in many studies (Table 3)[33]. After liver transplantation, food consumption progressively normalises and contributes to restoring nutritional status and body composition primarily in those patients who were more depleted.

There are few published studies concerning the modifications of the nutritional status after liver transplantation. However, multiple reports have suggested that muscle depletion is not reverted in the first year after liver transplant[34-36]. A better knowledge of the modification of nutritional status that occurs after liver transplantation could be the departing point for the application of dietary regimens or physical activity programmes targeted at improving nutrition in these patients.

The aim of this review is to examine the recent literature concerning the modifications of nutritional status after liver transplantation. Data concerning the impact of malnutrition on the outcome of liver transplantation were evaluated.

RESEARCH

Bibliographic searches were performed using PubMed and Embase for the following words (all fields): "nutrition" (MeSH) or "malnutrition" (MeSH) or "nutritional status" (MeSH) or "protein depletion" (MeSH) or "sarcopenia" (MeSH) or "muscle wasting" (MeSH) and "liver transplantation" (MeSH) or "liver transplant" (MeSH) or "end stage liver disease". The reference lists in the studies identified during electronic searching were hand-searched to identify additional relevant studies for inclusion in this review. Eligible studies for the review included those that were published as full papers in peer-reviewed journals between 1993 and 2013; however, older studies were utilised, when needed, to support the information concerning the physiopathology of malnutrition in liver disease. Studies published in non-English language were excluded; these non-English studies represented only a small percentage (< 5% of total), and, therefore, these excluded studies do not constitute a relevant bias. Preference was given to studies that presented original data, rather than review studies.

MODIFICATION OF NUTRITIONAL STATUS AFTER LIVER TRANSPLANTATION

Whereas malnutrition is a common feature in end-stage

Table 1 Prevalence of muscle depletion in cirrhotic patients and related outcomes				
Ref.	**Patients (*n*)**	**Definition of muscle depletion**	**Prevalence of muscle depletion**	**Outcome associated with muscle depletion**
Merli *et al*[5], 1996	1053	Mid Arm Muscle Area < 5th *P*	26% 38% M; 8% F	Lower survival in Child A and Child B
Alberino *et al*[13], 2001	212	Mid Arm Muscle Area < 5th *P* Mid Arm Muscle Area < 10th *P*	25% 37%	Lower survival at 6, 12 and 24 mo
Alvares-da-Silva *et al*[14], 2005	50	Hand-Grip Strength 2 SDs below the mean value for the controls	63%	Higher rate of major complications
Campillo *et al*[15], 2006	396	Mid Arm Muscle Area < 5th *P*	53.2% Child Pugh A: 74.3% M, 22.2% F; Child Pugh B: 68.9% M, 35.2% F; Child Pugh C: 54.7% M, 21.9% F	No correlation with in-hospital mortality
Peng *et al*[16], 2007	268	Protein Index < 0.82 or 2 SDs below the mean protein index for the controls	51% 63% M; 28% F Child Pugh A: 72%; Child Pugh B: 43%; Child Pugh C: 42%	No outcome evaluated
Huisman *et al*[17], 2011	84	Hand-Grip Strength Mid Arm Muscle Circumference	67% 58%	Higher risk of complications
Fernandes *et al*[20], 2012	129	Mid Arm Muscle Circumference Hand-Grip Strength 2 SDs below the mean value for the controls	13.2% 69.3%	No outcome evaluated
Montano-Loza *et al*[18], 2012	112	Lumbar Skeletal Muscle Mass Index at CT scan ≤ 38.5 cm^2/m^2 in women and ≤ 52.4 cm^2/m^2 in men	40% 50% M; 18% F Child Pugh A: 13%; Child Pugh B: 55%; Child Pugh C: 32%	Increased 3 and 6 mo mortality
Tandon *et al*[19], 2012	142	Lumbar Skeletal Muscle Mass Index at CT scan ≤ 38.5 cm^2/m^2 in women and ≤ 52.4 cm^2/m^2 in men	41% 54% M; 21% F Child Pugh A: 0% M, 14% F; Child Pugh B: 42% M, 21% F; Child Pugh C: 72% M, 23% F	Increased mortality in cirrhotic patients awaiting liver transplantation
Merli *et al*[21], 2013	300	Mid Arm Muscle Circumference < 5th *P*	39%	Higher rate of hepatic encephalopathy

M: Male; F: Female; CT: Computed tomography.

liver disease, nutrition abnormalities are expected to revert when a new functioning liver is given to the patient. However, unlike other complications, a reverse of malnutrition and more specifically of sarcopenia is not a rule after liver transplant. Moreover, other features of malnutrition, such as overweight and obesity, may occur in liver recipients during long-term follow-up.

Modifications in body composition after liver transplantation: Sarcopenia overweight and obesity

In 1999, Keogh and co-authors applied dual energy X-ray absorptiometry to assess the changes in bone mineral density and body composition after liver transplantation. The timing of the evaluation of body composition in this study was extremely heterogeneous (range, 3-44 mo after surgery)[37]. While an overall reduction in bone mineral density was observed, body weight increased by 12% after transplantation due to an increase in the fat mass (from 24.1% ± 2.0% to 35.1% ± 1.8%) and a decrease in the fat-free mass (-5.7% ± 1.4%). Similarly, in a small group of 14 unselected patients undergoing liver transplantation, using sophisticated techniques for estimating body composition, a loss of total body fat was reported during the early postoperative period, which was fully regained at

3 mo. In the same group of patients, a depletion in skeletal muscle protein was present after 12 mo[34]. A failure to revert nutritional impairment during the first year after liver transplantation was also documented in a mixed population of 70 cirrhotic and non-cirrhotic patients transplanted between 1997 and 1999. In this retrospective study, 44% of patients were still classified as having some degree of malnutrition one year after transplantation. The presence of malnutrition was associated with a worse nutritional status before transplantation and fat stores (triceps skinfold thickness) remained inadequate in 70% of malnourished patients at the end of the first year[38]. In a prospective cohort study, cirrhotic patients who were severely malnourished while on the waiting list showed further deterioration at 3 mo after transplantation; however, they improved at 6 and 12 mo. Once again, primary changes were observed for fat mass (median triceps skinfold: basal 10.8 mm *vs* 15.2 mm, 12 mo, *P* = 0.03), whereas the parameters of muscle mass showed only minor variations (mid-arm muscle circumference: basal 23.4 cm *vs* 24.0 cm, 12 mo, *P* = 0.3)[35]. More recently, pre- and post-transplant abdominal muscle and fat area were evaluated in 53 patients, using abdominal CT. The patients were examined at a variable distance from liver

Table 2 Mechanisms that cause a reduction in food intake in patients with cirrhosis		
Reduced nutrient intake	Decreased appetite and anorexia	Unpalatable diet (sodium and water restriction for peripheral oedema and ascites, protein restriction for hepatic encephalopathy)
		Dysgeusia due to micronutrient deficiencies (zinc or magnesium)
		Anorexic effect caused by increased levels of proinflammatory cytokines (TNFα, IL-1β, IL-6) and leptin
	Nausea and early satiety	Tense ascites
		Gastroparesis
		Small bowel dysmotility
		Bacterial overgrowth
	Frequent compulsory starvation	Hospitalisation
		Invasive diagnostic procedures requiring fasting
		Gastrointestinal bleeding and endoscopic therapy

TNF: Tumor necrosis factor α; IL: Interleukin.

Table 3 Vitamins and trace elements deficiencies in patients with cirrhosis		
	Mechanism of deficiency	Primary consequences
Water soluble vitamins		
Complex B and	Dietary insufficiency	Wernicke's encephalopathy and Korsakoff dementia,
Vitamin C	Intestinal dysmotility	anaemia, asthenia, scurvy
Fat soluble vitamins		
Vitamin A (Retinol) and vitamin E	Dietary insufficiency	Risk factor for developing cancer, including
	Malabsorption for cholestasis or due to medications (*i.e.*, cholestyramine)	hepatocellular carcinoma, night blindness
Vitamin D	Dietary insufficiency	Osteopenia and osteoporosis
	Malabsorption for cholestasis or due to medications (*i.e.*, cholestyramine, steroids)	
Vitamin K	Reduced exposure to UV light	K-dependent coagulation factors deficiency (II, VII, IX, X)
	Dietary insufficiency	
	Malabsorption for cholestasis or due to medications (*i.e.*, cholestyramine)	
Trace elements		
Zinc	Dietary insufficiency	Contribution to impaired glucose tolerance and diabetes,
	Malabsorption (intestinal dysmotility)	precipitation of hepatic encephalopathy
	Diuretic induced increased urinary excretion	
Magnesium	Dietary insufficiency	Loss of muscle strength
	Malabsorption (intestinal dysmotility)	

UV: UltraViolet.

replacement (19.3 ± 9 mo). Of the 66% of sarcopenic patients before LT, only 6% had a reversal of sarcopenia, while 14 of the 20 patients who were not sarcopenic pre-LT developed sarcopenia de novo after LT[36].

Other studies have primarily focused on the increase in body weight and BMI after liver transplantation, which may lead to a diagnosis of obesity in some patients. A retrospective study in 597 patients transplanted between 1996 and 2001 found that by 1 and 3 years, 24% and 31% of the patients, respectively, showed a BMI > 30 kg/m^2[39]. However, it should be noted that several of the patients included in that study were already obese before transplantation. Considering only those patients who were not obese at the time of surgery, 15.5% and 26.3% became obese at 1 year and 3 years, thus indicating that overweight and obesity can be recognised as a likely burden after liver transplantation. Gender and the length of steroid therapy (more or less than 3 mo) were not found to be risk factors for the development of overweight and obesity. In a smaller study, 23 patients were followed for 9 mo after transplantation. At the end of the observation period, 87% were classified as overweight or obese due

to a significant increase in fat mass and a slight improvement in lean mass (arm muscle circumference)[40]. Similar results were shown in 17 liver recipients followed before transplantation and 12 mo after transplantation. A progressive weight gain characterised by a prevalent increase in fat mass was reported; at the end of the study, the rate of obese patients increased from 11.8% to 29.4%[41]. In a longer follow-up (*n* = 143 patients, 4 years after liver transplantation), 58% of the patients were observed to be overweight, and 21% were observed to be obese. The multilogistic regression analysis demonstrated that obesity after LT was predicted by a higher BMI before LT and a significant weight gain after LT[42]. Another study showed that in 42 long-term survivors studied at a distance ranging from 18 mo to 100 mo after successful liver transplantation, the mean BMI and the fat mass were significantly higher in transplanted patients compared to 39 patients with liver cirrhosis and a cohort of healthy controls[43]. Studies with a cross-sectional design, however, suffered several limitations: transplanted patients are observed at different times, and long-term survivors are only those patients who exhibited a better outcome after

transplantation, which represent a relevant selection bias.

All of these data suggested that despite the regain of liver function after liver transplantation and the improvement in body weight after surgery, the alterations in body composition may persist. In particular, muscle depletion seems to persist for at least 12 mo or more.

FACTORS THAT MAY INFLUENCE NUTRITIONAL MODIFICATIONS AFTER LIVER TRANSPLANTATION

Liver gut brain axis

The common hepatic branch of the ventral vagus is involved in important physiological functions[44-46]. The afferent and efferent fibres travelling in this branch are crucial for mediating the complex orchestra of biochemical, molecular, and neuronal signals from gut, liver and brain that influence food intake and nutrient homeostasis. The normal hepatic innervations and more specifically, vagus innervation, are lost during transplantation. It has been suggested that the isolation of the liver from the autonomic regulatory control may influence not only nutrient absorption and metabolism, glucose and lipids homeostasis but also appetite signalling and eating behaviour. All of these modifications may contribute to the body composition and weight changes observed in liver transplanted patients.

Diet

The majority of the published studies reported a significant increase in dietary intake when the patients were followed after liver transplantation. These changes are particularly evident in those patients following severe dietary restrictions or in those suffering from relevant gastrointestinal symptoms or anorexia before liver transplantation. We observed that calories improved from a median of 27 kcal/kg per day to 32 kcal/kg per day; $P = 0.007$ and proteins from a median of 0.8 g/kg per day to 1.3 g/kg per day; $P = 0.02$ (comparing dietary intake before transplantation and 12 mo after liver transplantation)[35]. Similar results were reported by Richardson *et al*[40] in 2001, who correlated the high rate of overweight or obesity in liver transplant patients with the increase in energy intake (from 1542 ± 124 kcal/d to 2227 ± 141 kcal/d), a higher consumption of both proteins and carbohydrates and an approximately doubled intake of fat (from 62 g/d to 102 g/d) compared to pre-transplant[40].

Energy metabolism

Modifications in energy metabolism have been involved in the changes of nutritional status after LT; however, studies reporting resting energy expenditure (REE) measurements have provided controversial results. During the early post-operative period (2-4 wk), several studies showed no significant changes in REE[47], whereas other studies found that REE was increased to 130%[48] or 142%[34] of the predicted values in the same period. Subsequently (6-12 mo), a persistent hypermetabolism

was reported at 6 mo by several authors[34], whereas others found a reduced REE at 9 mo after liver transplantation[40]. In the latter study, no correlation was observed between body composition, energy expenditure and the immunosuppressive regimen; however, those patients with a reduced energy expenditure showed the higher increase in fat mass. By extending the follow-up to 14 or 32 mo after transplantation, another study found that the increase in energy expenditure normalised only when insulin sensitivity was restored; however, no correlation was found with body weight changes[49].

Finally, in a more recent study, the large majority (76%) of patients investigated one year after LT were normometabolic[41]. Hypermetabolism after transplantation was significantly associated with hypermetabolism before LT and a higher cumulative dose of prednisone.

Immunosuppressive therapy

Immunosuppressive agents are known to exert metabolic effects, which may be implicated in nutritional changes and body composition modifications after LT. Corticosteroids need attention as they increase appetite and fat deposition and decrease fat oxidation; moreover, they are responsible for increased proteolysis and impaired protein synthesis[50,51]. Calcineurin inhibitors, such as cyclosporine and tacrolimus, may affect energy metabolism and muscle mass[51,52]. Cyclosporine was found to be an independent predictor of post transplant weight gain[53], whereas tacrolimus has been reported to increase energy expenditure[54]. Both cyclosporine and tacrolimus may contribute to the impairment of muscle growth and muscle regeneration by inhibiting calcineurin, which exerts its effects on skeletal muscle differentiation, hypertrophy, and fibre-type determination[52,55]. Other immunosuppressive agents, such as sirolimus and everolimus, negatively influence muscle mass by inhibiting the mammalian target of rapamycin complex, which is a key regulator of protein synthesis[56].

MOLECULAR MECHANISMS OF SARCOPENIA AFTER TRANSPLANTATION

The majority of the studies dealing with molecular mechanisms of sarcopenia in liver cirrhosis have investigated experimental animal models, such as portacaval shunted rats and biliary duct ligated rats[57-59]. Few studies have been performed in cirrhotic patients[60]; therefore, definite conclusions could not be drawn. Similarly, the data on the mechanisms of post-transplant sarcopenia are lacking. Interestingly, in 3 subjects who had muscle reduction after transplantation, the mRNA expressions of genes regulating ubiquitin proteasome proteolytic components were unaltered, whereas those of myostatin were significantly elevated[36]. These data suggest that an inhibition of muscle protein synthesis, induced by myostatin, instead of an increase in protein degradation, may play a pilotal role in the pathogenesis of post-transplant sarcopenia. More

Table 4 Relationship between nutritional status and outcome after liver transplantation

Ref.	Patients (*n*)	Parameters used for the assessment of nutritional status	Prevalence of malnutrition	Outcomes related to malnutrition
Pikul *et al*[64],1994	68	Subjective Global Nutritional Assessment	79%	Prolonged ventilator support Increased incidence of tracheostomy More days in intensive care unit and hospital
Selberg *et al*[65], 1997	150	Anthropometry Body composition analysis Indirect calorimetry	41%-53%	Decreased 5-yr survival after liver transplantation
Harrison *et al*[66], 1997	102	Anthropometry Dietary intake	79%	Higher risk of infections
Figueiredo *et al*[7], 2000	53	Subjective Global Nutritional Assessment Hand-grip strength Body composition analysis	87%	More days in intensive care unit Increased incidence of infections
Stephenson *et al*[68], 2001	99	Subjective Global Nutritional Assessment	100%	Increased blood product requirement More days in hospital
Shahid *et al*[28], 2005	61	Hand-grip strength Anthropometry	Not reported	No correlation
de Luis *et al*[69], 2006	31	Subjective Global Nutritional Assessment Body composition analysis Dietary intake	Not reported	No correlation
Merli *et al*[70], 2010	38	Subjective Global Nutritional Assessment Anthropometry Indirect calorimetry Dietary intake	53%	More days in intensive care unit and hospital Increased incidence of infections
Englesbe *et al*[71], 2010	163	Psoas muscle area (CT evaluation)	Not reported	Decreased 1-yr survival

CT: Computed tomography.

studies are warranted to elucidate the molecular mechanisms responsible for sarcopenia after liver transplant.

NUTRITION AND EXERCISE COUNSELLING

It is conceivable that interventional programmes including dietary and exercise counselling may, in part, correct or completely normalise the nutritional alterations occurring after LT. Specific diet and exercise programmes may prevent the tendency to become overweight or help to obtain an adequate recovery of muscle mass. However, few studies with this goal were conducted. A randomised trial of exercise and dietary counselling after liver transplantation has been recently published. In this study, 151 liver transplant patients were enrolled and randomised into exercise and dietary counselling or usual care. A total of 119 patients completed testing 2, 6 and 12 mo after liver transplantation. Testing included the assessment of exercise capacity through oxygen consumption (VO2) using spirometry, quadriceps muscle strength, body composition by DEXA and a nutritional intake evaluation. The exercise and dietary counselling group showed a greater increase in VO2 peak with respect to controls; however, both groups (exercise and dietary counselling and usual care) presented similar increases in body weight, fat mass and lean mass during the follow-up[61]. Although the dropout rate was small (20%), the authors emphasised that the intervention can be planned only in those patients for whom exercise and dietary counselling can be safely implemented; furthermore, adherence to exercise

and nutrition was rated low (37%). The life-style changes should be evaluated after a longer follow-up period.

NUTRITIONAL STATUS AND OUTCOME AFTER LIVER TRANSPLANTATION

Although the modification in nutritional status that occurs after liver transplantation represents a topic that warrants extensive investigation, much information is available concerning the role of nutritional status on the outcome of patients undergoing liver transplantation. Patients with liver disease and malnutrition suffer a higher risk of complications and mortality after surgery[26,62]. Similar findings have been reported in patients undergoing liver transplantation (Table 4)[28,63-71].

Several studies reported a greater need for blood products during surgery[68], a higher rate of infections[66,67,70] a longer postoperative hospital stay[68,70], and a lower postoperative survival rate[65] in liver recipients affected by severe malnutrition. Recently, the relevant role of malnutrition on survival after liver transplantation was confirmed in a study that focused on muscle wasting[71]. By measuring the cross-sectional area of the psoas muscle on CT scans in 163 liver transplant recipients, a strong association was found between the psoas area and post-transplant mortality (HR = 3.7 per 1000 mm^2 decrease in the psoas area; $P < 0.0001$). The authors suggested that the objective measures of frailty, such as muscle wasting, may have the potential to inform benefit-based allocation models and may help optimise liver transplant outcome.

In contrast, other studies failed to show a correlation

between the nutritional status and the post-transplant outcome[28,69]. In these latter studies, surgical risk, donor risk index, and immunosuppressive therapy could have played a major role in the outcome of liver transplantation and might have blunted the influence of the recipient's nutritional status.

As muscle wasting is a well-known risk factor that contributes to increasing costs for morbidity and mortality after major surgery in the general population[72], the specific role of liver disease in this setting might be questioned. Undoubtedly, sarcopenia occurs more frequently in liver disease than in the general population. Furthermore, post-surgical one-year survival was found to be 87% in sarcopenic non-cirrhotic patients[72], but only 49.7% in sarcopenic cirrhotic patients who are undergoing liver transplantation[71].

Controversies exist concerning the influence of obesity on the outcome of liver transplantation. A higher rate of wound infection was reported in severely obese patients (BMI > 35 kg/m^2) who undergo liver transplant[73]. Additionally, these patients progressed more frequently to early death from multisystem organ failure. These results have been confirmed by analysing a large database including 18.172 transplanted patients, which demonstrated that primary graft non-function, and in-hospital, 1-year and 2-year mortality were significantly higher in the morbidly obese patients (BMI > 40 kg/m^2)[74,75]. A similar study, using the National Institute of Diabetes and Digestive and Kidney Disease liver transplantation database, found no significant difference in survival across all BMI categories after the BMI correction for ascites[76].

In conclusion, alterations in nutritional status and muscle depletion occur frequently in patients with end-stage liver disease. After liver transplantation, the recovery of muscle mass is challenging. Close monitoring of the modifications in the nutritional status and body composition in liver recipients will help to identify patients at risk for malnutrition or obesity after transplantation. Additional large-scale interventional studies are needed to evaluate whether physical and nutritional interventions after liver transplantation are capable of improving body composition and muscle function.

Malnutrition and severe obesity seem to affect the prognosis of these patients and have an impact on morbidity and mortality after liver transplantation. Recently, sarcopenia has been proposed to be an objective and valid prognostic index of mortality during and after liver transplantation, signalling the importance of severe muscle depletion in the clinical outcome of cirrhotic patients.

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TOPIC HIGHLIGHT

Using old liver grafts for liver transplantation: Where are the limits?

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Abstract

The scarcity of ideal liver grafts for orthotopic liver transplantation (OLT) has led transplant teams to investigate other sources of grafts in order to augment the donor liver pool. One way to get more liver grafts is to use marginal donors, a not well-defined group which includes mainly donors > 60 years, donors with hypernatremia or macrosteatosis > 30%, donors with hepatitis C virus or hepatitis B virus positive serologies, cold ischemia time > 12 h, non-heart-beating donors, and grafts from split-livers or living-related donations. Perhaps the most practical and frequent measure to increase the liver pool, and thus to reduce waiting list mortality, is to use older livers. In the past years the results of OLT with old livers have improved, mainly due to better selection and maintenance of donors, improvements in surgical techniques in donors and recipients, and intra- and post-OLT management. At the present time, sexagenarian livers are generally accepted, but there still exists some controversy regarding the use of septuagenarian and octogenarian liver grafts. The aim of this paper is to briefly review the aging process of the liver and reported experiences using old livers for OLT. Fundamentally, the series of septuagenarian and octogenarian livers will be addressed to see if there is a limit to using these aged grafts.

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Key words: Old liver donors; Liver transplantation; Aging liver; Liver graft; Liver disease; Aging; Donor management; Septuagenarian donors; Octogenarian donors

INTRODUCTION

Orthotopic liver transplantation (OLT) is the treatment of choice for patients with end-stage chronic liver diseases, acute liver failure, and certain metabolic liver diseases. The excellent results of OLT have led to an increasing number of patients on the waiting list, while the number of liver donors remains stable. Thus, the main limitation factor for OLT is having access to a liver graft. Moreover, the best results are obtained using ideal liver grafts that are defined as those obtained from donors younger than 40 years, trauma as the cause of death, brain death, hemodynamic stability at the time of procurement, and absence of steatosis, chronic liver disease, and transmission disease[1]. However, the ideal graft is becoming less and less frequent, mainly due to a progressive and dramatic reduction in traffic accidents. According to the Spanish

Liver Donor Registry, during the year 2000 the rate of donors between 15 and 45 years old was 40.6% versus 20.9% during the year 2012[2]. This liver organ shortage has led liver transplant teams to expand the donor pool using so-called marginal donors, a not well-defined group which mainly includes donors > 60 years, donors with hypernatremia, steatosis greater than 30%, or positive serologies for hepatitis C virus (HCV) or hepatitis B virus (HBV), livers with a cold ischemia time > 12 h, non-heart beating donors, and grafts from split-liver and living-related donations[3-11]. The most frequent and practical measure to augment the liver donor pool, and thus to reduce waiting list mortality, is to increase donor age[4,12-16]. However, the use of older livers for transplantation is subject to debate because several authors reported a negative impact of increased donor age on survival after OLT[17-20]. On the other hand, other transplant groups have found similar patient and graft survival rates using liver grafts older than 60 and even older than 70 and 80 years[13,15,21-26]. In an attempt to clarify the influence of the aged liver donor on the results of OLT we will review this issue in the literature especially regarding donors older than 70 years, and establish as accurately as possible if there is an age limit for utilizing a liver graft.

AGING PROCESS OF THE LIVER

Aging is characterized by normal progressive declines in functions that, cumulatively, diminish the capacity of cells and organs to respond to intrinsic and extrinsic stimuli. Functional changes that develop with aging should eventually lead to significant alterations in clinical practice. The synthetic, excretory and metabolic changes of liver function are potentially affected by aging and these effects may have clinical relevance[27]. Although this aging process does not cause death, it appears to contribute to the onset of diseases, including liver pathologies[28]. The major age-related changes in the liver are a reduction in mass and blood flow. However, the main differences and consequently the major advantages with respect to other organs are the maintenance of a good functional reserve, regenerative capacity, and large blood supply, all of which support the use of older donor livers for OLT[29,30]. Experimental findings in rodents, related with the aging process are generally very difficult to extrapolate to humans.

Morphologic changes

The old liver tends to be smaller and dark-colored, and generally suffers brown atrophy (brownish aspect), an appearance attributable to the increased accumulation of lipofuscin (highly oxidised insoluble proteins) and fibrous thickening of Glisson capsule[30-32]. There are few macroscopic and microscopic changes in the liver with aging, and the most widely recognized alteration is a decrease in weight[27]. In healthy people, the liver accounts for about 2.5% of the total body weight until about 50 years old. After that, the liver becomes gradually smaller, so that by the age of 90 it represents about 1.6% of total body weight[33]. The decrease in hepatic weight parallels a reduction in body weight[27]. There are other gradual changes such as in shape, moulding the liver with other organs or structures (ribs), and acquiring ridges and bosses on its surface[33].

Morphometric and ultrastructural changes

A 60% thickening of the endotelial lining and an 80% decline in the number of endothelial cell fenestration with increasing age was reported in a study that examined surgical and postmortem samples of human livers[34].

There are several morphological changes of hepatocytes associated with aging, such as an increase in mean volume and greater variance in the size of liver cells, a decrease in the number of hepatocytes, and increase in the size of liver cell nuclei and the volume of nuclear DNA in proportion to nuclear size. There is also an increased aneuploidy and a decreased number of mitochondria, but an increase in mitochondrial volume[35]. These morphological changes suggest that the liver cells in advanced old age are in a hyperfunctioning state possibly trying to compensate for the decline in absolute cell number[33]. In liver biopsy samples, of both healthy subjects and subjects with chronic liver disease, a progressive decline in telomere length with increasing age has been observed[36]. Recently changes in the hepatic sinusoid with old age have been identified that probably contribute to the substantial age-related changes in liver function[37].

Blow flow and volume changes

In the elderly population, there is an approximately 30% loss of liver volume and hepatic blood flow between the ages of 30 and 100[31,38].

This process starts at 25 years, at a rate of 0.3%-1.5% per year[39], and it would be expected that the liver blood flow of a 65-year-old is expected to be 40%-45% less than that of the same person at 25 years old[40]. A decrease in liver volume and liver blood flow with aging may be a major component of age-related alterations in the liver, leading to a fall in the clearance of many drugs whose pharmacokinetics have been found to be altered with age[31]. Atherosclerotic occlusive disease of visceral arterial branches of the abdominal aorta (celiac trunk and branches, mesenteric and renal arteries) occurs in 2.6% of all cases, and tends to be localized in the proximal or mid-proximal portions of the arterial bed; these lesions can be surgically amenable, but not in the occasional case where atherosclerosis is located in the distal portion of the bed[41] where the hepatic artery may be affected[42,43].

Synthetic and functional changes

The rate of total protein synthesis was 37% less in the 69-91 than in the 20-23 year old population, and the hepatic synthesis of clotting factors is also presumably impaired in the older patients[33].

It seems that the routine biochemical liver function tests (serum bilirubin, alkaline phosphatase and transaminase levels) do not alter with increased age, and are in

reality more a reflection of liver damage than a marker of poor function[29,33].

A fall in functional hepatic mass may be the most important change in the liver during normal aging, but that liver cells are little changed with age alone[29]. It has been suggested that aging has a limited effect on liver functions but more on its response to extrahepatic factors[44], disease states or increased metabolic demands to which elderly people may have an impaired ability to respond[29,33]. Some hypotheses state that, while enzymes responsible for normal metabolism or detoxification are adequate in the aged liver, the system is unable to respond to the increased stress of an external hepatotoxic agent[33]. Moreover, aging appears to compromise liver regeneration by influencing several pathways, the result of which is a reduction in the rate of regeneration, but not in the capacity to restore the organ to its original volume[45]. The aging process does not increase the susceptibility to hypoxia-reoxygenation injury in the rat liver, and although one should be cautious when extrapolating data on aging from rats to man, this finding lends additional support to the increasing use of older livers for OLT in humans[46].

ASSESSMENT AND MANAGEMENT OF THE LIVER DONORS

The definition of an ideal allograft is different from that of an ideal donor. Thus, the ideal allograft may be influenced by some variables that are introduced after procurement such as prolonged cold ischemia time (CIT), or partial or split-liver grafts[47]. Donors are generally considered marginal or extended criteria donors if there is a risk of initial poor function (IPF) or primary nonfunction (PNF). There is a lack of agreement on the definitions of primary dysfunction, IPF and PNF. It has been suggested that primary dysfunction can be used to describe all grafts that function poorly in the post-OLT period (*e.g.*, PNF and IPF). PNF refers to liver grafts that fail to support life in the early post-OLT period (first week) and die or required a retransplant for the patient to survive. On the other hand, IPF is defined as an aspartate aminotransferase (AST) level of more than 2000 IU/L, prothrombin time more than 16 s and ammonia level of more than 50 μmol/L on post-OLT days 2 to 7 in a context of graft supporting life[48].

Although marginal liver grafts may not be optimal, they are a viable alternative to dying while candidates are on a waiting list for OLT[7]. At present, there is not a clear and established definition of a marginal liver donor. Among the most important donor characteristics that may influence the development of PNF or IPF in the recipient are increasing age, prolonged ischemia, hypotension and inotropic support, gender mismatch, non-heart-beating donors, and steatosis[7,49,50]. A literature review revealed at least 13 donor variables that may be associated with poor graft survival and increased recipient mortality. These variables were donor age, race, gender, weight, ABO status, cause of brain death, hospital stay, pulmonary insufficiency, vasopressor use, cardiac arrest, alterations of blood chemistry, prolonged CIT, graft steatosis, hypernatremia, donation after cardiac death, and positive serologies for HBV or HCV[1,3,7]. However, there is a great variability in the number and type of variables included in the term extended criteria. Thus, seven donor characteristics were identified using Cox regression models that independently increase the risk of graft failure: donor age over 40 years (particularly over 60 years), donation after cardiac death, and split/partial grafts were strongly associated with graft failure, while black race, less height, cerebrovascular accident and other causes of brain death were less but still significantly associated with graft failure[1]. Other research regarding extended criteria found donor age > 55 years, donor hospital stay > 5 d, cold ischemia time > 10 h, and warm ischemia time > 40 min as predictive risk factors of poor outcome after OLT[51]. With the aim to analyze the influence of several marginal criteria in donors, a marginal liver score was elaborated with the following variables: donor > 60 years, ICU stay > 4 d, CIT > 13 h, hypotensive episodes < 60 mmHg for > 1 h, bilirubin > 2.0 mg/dL, alanine aminotransferase (ALT) > 170 U/L, and AST > 140 U/L (each variable assigned a value 1), use of dopamine doses > 10 μg/kg, and serum sodium > 155 mEq/L (each variable assigned a value of 2). Recipients who received marginal livers with a score of 3 or more showed significantly lower graft survival and delayed graft function[52].

Evaluation and support of older liver donors

Between 70%-88% of donors older than 70 years die because of cerebrovascular disease[13,23,53-55]. When brain death is declared and liver donation is being considered, the primary goal is maintenance of the organ´s viability. Thus, the measures for the protection of the liver graft must be as follows: resuscitation in the event of cardiac arrest, maintenance of an effective circulation to prevent ischemic injury, therapy of hypovolemia to maintain a systolic blood pressure (SBP) or central venous pressure above 10 cm H_2O, blood transfusion if hematocrit is less than 25%, oxygenation to maintain P_aO_2 between 70-100 and O_2 saturation at 95%, prevention of infection and maintenance of normothermia and diuresis greater than 1 mL/kg per hour. A SBP between 80-100 mmHg maintained during more than one hour has been considered a criterion of a marginal liver donor by some authors[13,56]. When SBP is less than 100 mmHg, dopamine infusion is indicated to increase mesenteric and renal blood flow. Initially the dose is 2-5 mcg/kg per minute, bearing in mind that renal function impairs and that acute tubular necrosis can develop when the dose of dopamine is > 10 mcg/kg per minute. Several groups define a dose of dopamine > 15 mcg/kg per minute as a marginality criterion[13,49,56]. The use of a dopamine dose > 15 mcg/kg per minute associated with SBP < 90 mmHg increases significantly the grade of graft preservation injury[49]. Cardiac arrest during a period of 15 min does not significantly affect PNF

or graft function[57], although one German team does not use graft livers from septuagenarian donors with cardiac arrest[58].

Prolonged ICU stay of donors can modify post-transplant liver function due to hemodynamic, hormonal and nutritional alterations and other alterations produced by vasopressor drugs[59]. According to some authors the rates of PNF and graft dysfunction increase with a mean ICU stay of > 3 d[60], while others find for the same ICU period only find transaminase values higher than 2000 IU/L but without affecting graft survival[49]. More recently, a study considered an ICU stay of > 4 d as a marginal criterion due to the associated higher rate of preservation injury[56]. According to several series using liver grafts over 70 years, mean ICU stay is < 3.5 d[13,23,26,53,55,58,61]. The deleterious effect of hypernatremia (peak serum sodium > 155 mEq/L) on graft function is thought to be a result of cell swelling and exacerbation of reperfusion-mediated injury[7]. The presence of hypernatremia has been associated with marked graft dysfunction[62,63], and even with significantly lower 1-month graft survival[64]. However, donor serum sodium showed normal mean values in five series of donors older than 70 years[23,53,55,58,61,65]. The elevation of liver enzymes [glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and gamma glutamyl transpeptidase (GGT)] in donors may reflect a process of cytolysis, cholestasis, hypoperfusion due to hypovolemia, or cardiac arrest, and liver enzymes values can rise to 400 IU/L during short periods of ischemia or asystolia[66]. The presence of values of GOT > 150 IU/L and GPT > 170 IU/L[56], and of GGT > 100 IU/L in donors older than 70 years have been considered as marginal criteria[13]. Several reports using donors older than 70 years showed mean values of GOT, GPT and GGT within normal limits[26,30,53,55,58]. In the absence of hepatobiliary disease, the presence of hyperbilirubinemia in the donor can be due to hemolysis, and it is not demonstrated that bilirubin > 2 mg/dL is associated with lower graft survival or graft dysfunction in comparison with lower bilirubin values[49]. Mean bilirubin values in several studies using liver donors older than 70 years range between 0.7 and 0.95 mg/mL[13,23,53,55,58]. In comparative series that analyze donors older than 70 years, liver function tests are more favorable in older donors, a finding that reflects the meticulous selection of older donors[23,30,53,55] in order to counterbalance the risks associated with the aging process[55].

In the process of evaluation of donors older than 70 years an ultrasonography is recommended to exclude benign or malignant hepatobiliary diseases, liver steatosis, and other abdominal tumors. During the procurement procedure it is necessary to explore the abdominal cavity to confirm the absence of tumors or abscess. A liver biopsy is highly recommended in octogenarian[30,53] and in septuagenarian liver donors to exclude liver disease (steatosis, cholestasis, hepatitis, or fibrosis).

Steatosis of liver graft
A liver is considered steatotic when the lipid content exceeds 5% of the body weight, and the reported incidence is between 9%-26% among the liver donor population[67,68]. Steatosis is more frequent among old donors, and has been attributed to alcohol intake, obesity, malnutrition, and diabetes[69,70].

Steatosis is classified as mild (10%-30%), moderate (30%-60%), or severe (> 60%)[69], but it is believed that steatosis will disappear after OLT.

Steatotic liver grafts are more prone to developing preservation injury, and a short ischemic injury is recommended to prevent preservation injury[70,71].

We observed a higher rate of overall steatosis in donors older than 60 years at the expense of macrosteatosis[55], although the liver grafts with any degree of isolated microsteatosis can be safely used, except for the risk of initial dysfunction, because it does not adversely affect patient or graft survival[5,72,73]. The experience of the surgeon is essential for the evaluation of the presence of steatosis during liver procurement and it must be confirmed by microscopic examination. It has been confirmed that the combination of increased BMI, elevation of ALT, presence of type II diabetes, history of heavy alcohol consumption, and ultrasonography signs of steatosis can identify steatosis > 30%[74]. OLT with livers with macrosteatosis < 30% has similar results as OLT with non fatty livers, assuming there are no other concomitant donor or recipient risk factors[3,7]. The implant of a liver graft with moderate-severe macrosteatosis precipitates severe ischemia-reperfusion injury and puts a patient at increased risk of initial poor graft function[48,67,73,75-77], PNF[69,70,75] and lower graft and patient survival[73]. It has been reported that liver grafts with macrosteatosis > 30% can be safely used in low-risk patients, but should be avoided in patients with high model for end-stage liver disease (MELD) scores[78]. Other investigators report a comparable 3-year patient survival in a control recipient group and a study group showing severe macrosteatosis (> 60%), and the authors conclude that severely steatotic livers should be considered for OLT at least in low-risk patients, but that short ischemia times must be observed and perioperative management must be optimized when using steatotic liver grafts[76]. In a recent report using liver grafts with severe macrosteatosis from donation after cardiac death (DCD), it was concluded that these grafts should only be considered for OLT in selected patients with preserved liver function (e.g., sclerosing cholangitis) and favorable MELD scores, without the presence of additional risk factors such as livers from DCD[77].

In series of donors older than 70 years, the incidence of steatosis was between 16% and 50% of cases, and hepatocytes was involved in less than 30% of all reported cases[13,23,53-55,65]. All series of octogenarian donors avoid the use of liver grafts with macrosteatosis > 30%[14,24,30,53,58,79,80].

Ischemia times
Prolonged CIT of liver causes a microvascular injury, called ischemia-reperfusion (IR) injury, which can lead to PNF or IPF and increased rejection and morbidity.

IR injury of liver grafts develops in four stages: pre-preservation injury in the donor, cold preservation, rewarming, and reperfusion injury. The incidence and grade of IR injury may be affected by several factors related to donor medical history, such as use of donors older than 60 years, prolonged ICU stay, alcohol intake, drug abuse, liver steatosis, hemodynamic instability after brain death, hypotension, high doses of inotropic drugs, prolonged CIT, and surgical trauma during the procurement process[7,56]. In other series comparing donors younger and older than 65 years, no significant impact was observed of donor age or CIT (< 8 h and ≥ 8 h) on the incidence of IR injury, short-term liver function, and 1-year patient and graft survival[81]. However, it is known that recipients of old livers have a greater sensitivity to IR injury, as reflected by a notable cholestatic pattern after OLT[23,53,82]. Furthermore, CIT of older donors must be kept as short as possible to obtain good liver function after OLT[13,53,55,83]. Thus, in eight series that included septuagenarian donors, the mean CIT was between 5 and 8 h[23,26,53-55,58,61,65], and only one series showed a mean CIT of 9 h[13]. Older grafts with a CIT > 8 h are at much greater risk for failure; with a CIT > 12 h the risk approximately doubles[84].

Prolongation of warm ischemia time (WIT) increases cold ischemia injury and consequently impairs post-transplant liver function[85]. Deleterious effects on patient and graft survival have been reported when WIT was longer than 40 min[51], and on graft survival alone when WIT was greater than 45 min[86], but usually most series of donors older than 70 years report a mean WIT between 45 and 65 min[13,53,55,58,61].

Allocation of older donors to recipients

The MELD score has been used as a measure of mortality risk in patients with end-stage liver disease and it is deemed suitable for use as a severity index for guiding organ allocation priorities[87]. Mortality on the waiting list increases in direct proportion to the MELD score at the time of listing[88]. The implant of marginal livers into suboptimal recipients constitutes a bad combination. At present, there is a tendency to allocate livers from old donors to stable patients[7,58,71]. Moreover, an octogenarian donor liver can be implanted into a sexagenarian recipient, but many groups would be reluctant to accept such a liver for a child[89].

Having in mind that the sickest patients must be transplanted first, livers from high-risk donors should be used for low-risk recipients only, whereas high-risk recipients should only be transplanted with low-risk organs[90]. More specifically, younger donor livers should be preferentially transplanted into HCV-positive recipients and livers from older donors into older HCV-negative recipients. This preference is based on the observations that the worse patient and graft survivals correlated with highest HCV recurrence, when liver grafts from donors older than 40-50 years[91-97], or older than 70 years are transplanted into HCV-positive recipients[7,14,55,79,80]; ex-

ceptions are the series of Doyle *et al*[98] and our series[55], where no significant differences in terms of 1-, 3-, and 5-year patient and graft survival were observed between HCV-positive recipients of liver grafts younger than 60 years and HCV-positive recipients of liver grafts older than 60 years. However, in our study there was a tendency towards decreased patient survival at 5 years, taking into account that our rate of HCV-positive cirrhosis was significantly higher in recipients of donors older than 60 years[55]. In some series HCC and ethylic cirrhosis were the most frequent indications for using donors older than 70 years[13,27,58,99,100].

Similar MELD scores have been found in several series of recipients transplanted with livers from donors older than 70 years[26,55,58].

POST-TRANSPLANT EVOLUTION OF OLD LIVERS AND COMPLICATIONS

A correlation between the incidence of PNF/IPF and older donors has been pointed out[48,60]. The incidence of PNF was reported to be between 2.7% and 8% in 6 series of recipients of donors older than 70 years[13,54,61,65,100], whereas in 4 other series there was not any case of PNF[23,26,55,58].

The non-rejection-related cholestasis pattern after OLT was significantly more frequent in recipients of donors older than 70 years in comparison with recipients of younger donors[23]. Synthesis parameters (serum albumin, partial thromboplastin time) were normalized at one week after OLT, while liver function tests (ALT, AST), and bilirubin showed normal values at three months post-OLT[99]. In our experience, the serum values of GOT, GPT, GGT, and bilirubin, at one month post-OLT, were similar in recipients of donors younger and older than 70 years. Moreover, prothrombin rate and serum albumin levels were significantly lower on the 30th day after OLT in recipients of donors older than 70 years[55], and these findings were attributed to a decrease in protein synthesis[101] and coagulation factors that run parallel to the liver aging process[102].

Intensive care unit stay (between 4 and 7 d) and hospital stay (between 20 and 25 d) were similar for recipients of donors younger or older than 70 years[23,53,55]. Likewise, the rates of acute and chronic rejection did not differ between recipients of donors older and younger than 70 years[23,53,58,100]. In several series there were no differences in the rate of biliary and hepatic artery complications[23,53,58], but recently it was emphasized that ischemic-type injury rates increase significantly with donor age over 70 years[100]. A recent series from united network for organ sharing database reported that the risk of graft loss from hepatic artery thrombosis (HAT) increased progressively with each decade of donor age > 50 years, such that a 61% increased risk of HAT-related graft loss was associated with use of donors older than 70 years[103]. More recently, an experience with donors older than 70 years showed a low incidence of HAT (4.7%), and im-

Ref.	Cases > 60 or > 65 yr (*n*)	Donor mean age (yr)	Cold ischemic time (h)	Recipient mean age (yr)	Primary non-function	Patient survival (yr)	Graft survival (yr)
Marino *et al*[12]	54 > 60	65.2	12.8	53.8		2-yr: 62%	2-yr: 43%
Washburn *et al*[106]	29 > 60	63.7	10.6		6.7%	1-yr: 58.6%	1-yr: 44.8%
Grande *et al*[107]	40 > 60	68	6.5		5%	1-yr: 82%	1-yr: 77%
						5-yr: 75%	5-yr: 66%
Rodríguez *et al*[22]	100 > 60	69	4.1	54	1%	1-yr: 82%	1-yr: 77.8%
						5-yr: 74.5%	5-yr: 71.4%
Neipp *et al*[111]	67 > 60	65	10.3	49	12%	1-yr: 79%	1-yr: 68%
						5-yr: 62%	5-yr: 53%
Moore *et al*[20]	35 > 60					5-yr: 48%	5-yr: 35%
Anderson *et al*[15]	91 > 60			54	3.3%	1-yr: 86.8%	1-yr: 82.4%
						5-yr: 67.6%	5-yr: 62.5%
Rauchfuss *et al*[110]	54 > 65		8.4			1-yr: 70%	1-yr: 70%
Martins *et al*[81]	50 > 65	73.9	7.3	57.6	4%	1-yr: 78%	
Jiménez-Romero *et al*[55]	125 > 60	69.1	6.1	51.2	0.8%	1-yr: 80.7%	1-yr: 78.2%
						5-yr: 68.5%	5-yr: 65.1%

Table 1 Series of orthotopic liver transplantation with liver grafts > 60 or > 65 years old

proved results were attributed to more appropriate technical management, whereas the presence of anatomical variations and use of jumping grafts were independent predictors of HAT[104].

The incidence of infections was similar[58] or even lower in recipients of liver donors older than 70 years[55]. Most reports have found similar rates of retransplantation comparing recipients from 70-year-old donors and younger donors[13,23,55,58,100].

Most common causes of mortality in recipients of donors older than 70 years are medical complications, *de novo* tumors, and cirrhosis due to HCV recurrence[53,55].

PATIENT AND GRAFT SURVIVALS USING OLD LIVERS

Liver grafts younger than 70 years

The use of aged liver grafts has progressively increased during the past decade due to improving results related to better management and procurement techniques of liver donors, and better hepatectomy and implant techniques in the recipients. In the nineteen nineties livers from donors older than 50 years were considered to be aged livers. However, several comparative series with younger donors demonstrated no significant differences regard to the rates of primary graft failure, retransplant, and patient and graft survivals, leading to the conclusion that liver grafts older than 50 years can be safely used for transplant[71,82,105].

The first two comparative series using liver grafts older than 60 years showed significantly lower 1-year graft survival[106], and 2-year graft survival in recipients of older livers, which was attributed to the more frequent ischemic injury in this group[12]. In two posterior reports comparing recipients of livers older and younger than 60 years, the rates of patient and graft survival, primary graft failure, and graft dysfunction were similar, but the mean CIT ranged between 5 and 6.3 h[22,107], significantly less than the previous series with a CIT of 12.8 and 10.6 h, respectively[12,106]. It has been established that prolonged

CIT impairs liver graft function, and when CIT is longer than 14 h the graft preservation injury doubles[56]. In an analysis of liver transplants from the Scientific Registry of Transplant Recipients, donor age over 60 years was the strongest risk factor for graft failure[1]. Other small series obtained significantly worse results using donors older than sixty years[20], but more recently larger series of 91 OLT[15] and 125 OLT[55] confirmed no significant differences when comparing the use of donors older and younger than 60 years.

In a comparative series of five groups divided according to donor age categories (donors < 50 years; donors between 50-59 years; donors between 60-69 years; donors between 70-79 years; and donors ≥ 80 years), the predictors of poor graft survival were donor age between 60-79 years, HCV-positive recipients, MELD score ≥ 25, and emergency OLT[26].

In two comparative studies using liver donors older and younger than 65 years, graft survival was lower in the group of recipients of older donors, and the rate of graft dysfunction was higher when the grafts presented steatosis[108,109]. However, two more recent studies did not find any significant differences in patient and graft survival using liver grafts younger or older than 65 years[81,110].

These and other experiences using donors older than 60 years are shown in Table 1[12,15,20,22,55,81,106,107,110,111].

Liver grafts older than 70 years

Most authors have established that the use of liver grafts from septuagenarian donors *per se* is not a contraindication for their utilization in OLT[13,23,26,54,55,58,99,100,112]. However, some authors reported significantly worse patient and graft survival when they used liver grafts older than 70 years[19,61,65] (Table 2).

The way to get good results when using liver grafts older than 70 years is to make a good donor selection, avoiding, as far as possible, the use of grafts with marginal donor criteria that are known to be associated with IPF and PNF of the graft. It is also important to avoid recipient risk factors (advanced age, obesity, renal dis-

Table 2 Series of orthotopic liver transplantation with liver grafts older than 70 years

Ref.	Cases (*n*)	Donor mean age (yr)	Cold ischemic time (h)	Recipient mean age (yr)	Primary non-function	Patient survival (yr)	Graft survival (yr)
Emre *et al*[13]	36	73.5	9	55	5.5%	1-yr: 91%	1-yr: 85%
Kim *et al*[54]	25	74	7.6	49	8%	1-yr: 95.4%	1-yr: 82.7%
						3-yr: 89.8%	3-yr: 71.7%
Gastaca *et al*[23]	55	-	5	-	0%	1-yr: 93.8%	1-yr: 92.6%
						3-yr: 90.6%	3-yr: 89.4%
Borchert *et al*[99]	41	73.4	8.9	50.9	2.4%	1-yr: 91%	1-yr: 86%
						3-yr: 83%	3-yr: 81%
						5-yr: 77%	5-yr: 75%
Segev *et al*[25] (UNOS)	1043	74.8				3-yr: 81.2%	3-yr: 74.9%
Cescon *et al*[26]	111	-			7%	5-yr: 66%	5-yr: 62%
Fouzas *et al*[65]	17	73	7.2	57	11.8%	1-yr: 69.7%	
						3-yr: 57.5%	
						5-yr: 46.2%	
Lai *et al*[61]	28	74	6.4	57	3.6%	5-yr: 47%	5-yr: 40.7%
Sampedro *et al*[112]	24	78.3	3.7	53.9	0%	1-yr: 78%	
						5-yr: 63%	
Darius *et al*[58]	58	77	8	61	0%	1-yr: 90%	1-yr: 88%
						5-yr: 84%	5-yr: 79%
Jiménez-Romero *et al*[55]	50	75.7	6.1	51	0%	1-yr: 76%	1-yr: 73.9%
						5-yr: 62.9%	3-yr: 64.6%
							5-yr: 58.3%

UNOS: United network for organ sharing.

ease, HCV cirrhotic recipients, retransplant) related with increased graft loss and mortality[14,23,54,55,58,71,99,100]. When using liver grafts older than 70 years in preferred recipients (first time recipients over the age of 45 years, BMI < 35 kg/m^2, non-status 1 registration, CIT < 8 h, and either hepatocarcinoma or an indication for transplantation other than HCV cirrhosis), the results are similar to outcomes with younger liver grafts[25].

When using donors older than 70 years, 1-year patient survival varies between 66% and 95.4%, 3-year patient survival between 57.5% and 90.6%, and 5-year patient survival between 46.2% and 84%[13,23,25,26,55,58,61,65,99,112]. In addition, 1-year graft survival varies between 73.9% and 92.6%, 3-year graft survival between 64.6% and 89.4%, and 5-year graft survival between 40.7% and 79%[13,23,25,26,58,61,99]. It must be taken into consideration that some series excluded septuagenarian donors for transplant recipients with HCV cirrhosis, so that the results are better[58,99].

Liver grafts older than 80 years

Since the first reported case of successful use of an 86-year-old liver graft[113], several series of octogenarian liver grafts have been published[24,26,30,79,80] (Table 3). Moreover, other isolated cases of nonagenarian liver grafts were recently reported[114-116].

Cerebrovascular diseases are the causes of death of between 73% and 81.7% of octogenarian donors[26,53,80].

The general acceptance criteria of octogenarian liver grafts were: normal gross appearance and consistency, no alteration of liver function tests, hemodynamic stability with use of low doses (< 10 mcg/kg per minute) of vasopressors before procurement, ICU stay < 3 d, no relevant histological alterations in the pre-transplant biopsy, such as fibrosis, hepatitis, cholestasis, macrosteatosis >

30%), and short cold ischemia time (< 10 h)[30,53,79]. Liver biopsy during octogenarian donor procurement is generally recommended before accepting the use of the liver graft[14,26,30,79,80].

The reported rate of octogenarian grafts discarded is significantly higher than that of younger donors, and the principal reasons for graft refusal were moderate-massive steatosis, HCV cirrhosis and malignancies[53]. In series that compared octogenarian and younger donor characteristics, no significant differences were seen regarding ICU stay > 5 d, BMI ≥ 35 kg/m^2, use of norepinephrine, prevalence of steatosis, total bilirubin, alteration of liver function tests, serum sodium, hypotensive episodes or vasopressor use[26,53]. In our series[30], with ICU stays between 12 and 24 h, there was no cardiac arrest in any of our four donors, and the blood pressure was maintained above 90 mmHg with the use of up to 15 mcg/kg per minute of dopamine in three donors. With a CIT of less than 9 h all of our recipients attained a good early post transplant liver function. Thus, the current tendency for use of octogenarian donors is to minimize ICU stay (< 3 d), CIT (< 9 h), and steatosis[24,26,30,80] to prevent the development of ischemia-reperfusion injury that contributes to recurrence in HCV-positive recipients[117].

The worse outcome associated with the use of older donors for HCV-positive recipients has undergone a dramatic shift in the past years, so that nowadays octogenarian donor livers are mainly transplanted into patients with hepatocarcinoma and ethylic cirrhosis, avoiding OLT in viral C cirrhosis[26,79]. Thus, in this group the MELD score is higher than in recipients of younger donor livers where the rate of recipients who underwent OLT due to hepatocarcinoma is lower[26].

One-year patient survival ranges between 75%

Table 3 Series of orthotopic liver transplantation with liver grafts older than 80 years						
Ref.	Cases (*n*)	Donor mean age (yr)	Cold ischemic time (h)	Recipient mean age (yr)	Primary non-function	Patient survival (yr)	Graft survival (yr)
Jiménez-Romero et al[55]	4	85.7	5.5	50.2	0%	1-yr: 75%	1-yr: 75%
Nardo et al[53]	30	82.3	7.5	52.5	0%	1-yr: 80%	1-yr: 77%
Zapletal et al[24]	5		9.5	52	0%	1-yr: 100%	1-yr: 100%
Cescon et al[26]	41			52.5	0%	3-yr: 86%	3-yr: 81%
						5-yr: 86%	5-yr: 81%
Petridis et al[79]	10	83.5	5	57.4	10%	1-yr: 80%	
						3-yr: 40%	
Singhal et al[80] (UNOS)	197			58.5		1-yr: 81%	1-yr: 75.5%
						3-yr: 69.1%	3-yr: 61.2%

UNOS: United network for organ sharing.

and 100%, 3-year patient survival between 40% and 86%[24,26,30,53,79,80], and 5-year patient survival is 86%[26]. One-year graft survival varies between 75% and 100%, 3-year graft survival between 61.2% and 81%[24,26,30,53,79,80], and 5-year survival of 81%[26].

CONCLUSION

At the present time, there are enough studies regarding the use of sexagenarian and septuagenarian donors that demonstrate similar results in comparison with the use of younger donors. With respect to the use of octogenarian donors for OLT, experiences are less and shorter, but at least in Spain the utilization of such grafts is progressively increasing because of the necessity to expand the donor pool with the aim to decrease waiting list mortality. In order to get good results using old liver grafts with no age limit, careful donor selection must be performed (normal liver function, good hemodynamic and pre harvesting conditions, ICU stay < 72 h, CIT < 8 h, WIT < 1 h, macrosteatosis < 30%, absence of atherosclerosis in the hepatic artery, and absence of histological alterations in the biopsy), while avoiding recipient risk factors such as advanced liver disease (high MELD scores) or the presence of HCV cirrhosis frequently associated with higher HCV recurrence and additionally greater morbi-mortality. A liver biopsy should is advisable before accepting a liver graft older than 70 years, and also in doubtful cases of donors younger than 70 years.

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TOPIC HIGHLIGHT

WJG 20ᵗʰ Anniversary Special Issues (7): Liver transplant

Corticosteroid-free immunosuppression in liver transplantation: An evidence-based review

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Abstract

Thirty-six randomized controlled trials and two meta-analyses were reviewed. With respect to adult patients undergoing first orthotopic liver transplantation (OLT), steroid replacement resulted in fewer cases of overall acute rejection in the corticosteroid free-immunosuppression arm. Initial steroid administration for two weeks and early tacrolimus monotherapy is a feasible immunosuppression regimen without steroid replacement, although further investigations are needed in view of chronic rejections. No significant differences were noted between the treatment groups in terms of patient and graft survival independently of steroid replacement. Renal insufficiency, *de novo* hypertension, neurological disorders and infectious complications did not differ significantly among steroid and steroid-free groups. Diabetes mellitus, cholesterol levels and cytomegalovirus infection are more frequent in patients within the steroid group. With respect to diabetes mellitus and hypercholesterolemia, the difference was independent of steroid replacement. In relation to transplanted hepatitis C virus patients, mycophenolate mofetil does not appear to have a significant antiviral effect despite early reports. Male gender of donors and recipients, living donors, cold ischemia times, acute rejection, and early histological recurrence were related to the development of advanced hepatitis. There is sufficient scientific clinical evidence advocating avoidance of the ab initio use of steroids in OLT.

Core tip: Steroid replacement in orthotopic liver transplantation results in fewer cases of overall acute rejection in the corticosteroid free-immunosuppression arm. Tacrolimus monotherapy is a feasible immunosuppression regimen without steroid replacement, although further investigations are needed in view of chronic rejections. No significant differences were noted between the treatment groups in terms of patient and graft survival independently of steroid replacement. Male gender, living donors, cold ischemia times, acute rejection, and early histological recurrence are related to the development of advanced hepatitis. There is sufficient evidence advocating avoidance of the *ab initio* use of steroids in orthotopic liver transplantation.

INTRODUCTION

During the last decades, patients undergoing liver trans-

plantation have had favorable outcomes, mainly due to the evolution of immunosuppressive agents. The use of steroids is still considered the mainstay of immunosuppression following liver transplantation as they decrease the risk of rejection. Nevertheless, they are also related to a large number of side-effects, as well as the potential recurrence of hepatitis C virus (HCV). The most common indication for liver transplantation is chronic HCV infection, accounting for about 40% of all transplants performed in the United States[1]. Initially, the main concern after liver transplantation has always been the prevention of rejection, but current challenges also include preventing toxicity from anti-rejection (immunosuppressive) agents, while providing adequate immunosuppression to preserve optimal results[2]. The advent of new agents addresses the need for immunosuppression to be more specific and free of the long-term side-effects of steroids. Immunosuppressive protocols currently focus on the avoidance of steroids and the use of a combination of different agents that reduce their respective toxicities. Many authors believe that early withdrawal of steroids can be made safely[3,4], but the duration of steroid administration after liver transplantation and the initiation of steroid-free immunosuppression remain controversial.

A number of randomized control trials (RCTs) have been published concerning outcomes of steroid avoidance in liver transplantation. Two recently published meta-analyses (including RCTs published up to 2007) failed to draw robust conclusions owing to the heterogeneity of studies.

The different immunosuppressive regimens, varying follow-up periods, small sample sizes, and high percentages of failure of participants to undergo the allocated treatment were important bias factors in these meta-analyses.

Various sufficiently powered RCTs have been published in the last six years, addressing specific issues of steroid avoidance in liver transplantation.

The purpose of this review was to better define the role of the steroid-free immunosuppressive regimens in liver transplant recipients according to Evidence-based Medicine Levels of Evidence.

RESEARCH

With the intention of identifying suitable studies, the electronic databases Medline, Embase, Pubmed and the Cochrane Library were used to search for articles from 1990 to 2013 in the English language literature which integrated the subsequent terms and/or combinations in their titles, abstracts or keyword lists: Randomized controlled trials, double-blind, liver transplantation, steroids, withdrawal, glucocorticoids, prednisone, methylprednisone, orthotopic liver transplantation and allograft. Where it was appropriate the above-mentioned terms were used in "(MESH)" (Pubmed and the Cochrane Library) otherwise the terms were combined with "AND/OR" and asterisks. Furthermore, the abstracts from national and international conferences were searched using

online search engines corresponding to the particular conference.

After the initial screen additional criteria were imposed: (1) no less than one treatment group had early withdrawal or no steroid administration and a second treatment arm in which the patients received at least 3 mo of steroids; (2) study analysis was by intention to treat; and (3) studies of pediatric patients or both pediatric and adult population were excluded.

The two authors separately chose studies for inclusion and exclusion and reached consensus when they did not agree in the initial allocation. The subsequent variables were recorded: authors, journal and year of publication, country of origin, trial duration, participant demographics and data concerning rejection, adverse events, complications, follow up and survival.

CORTICOSTEROID-FREE IMMUNOSUPPRESSION IN LIVER TRANSPLANTATION

Thirty six RCTs[5-40] (some of which were updated versions of already published studies) and two meta-analyses[41,42] (including 19 and 21 of the above-mentioned RCTs, respectively) were reviewed. The evidence-based medicine levels of evidence and grades of Recommendation are shown in Tables 1 and 2. The baseline characteristics of the RCTs are summarized in Table 3. The strength and quality of the evidence is summarized in Table 4.

Adult patients undergoing first OLT for any indication

Rejection: Steroid replacement is presented with fewer cases of overall acute rejection in the corticosteroid-free immunosuppression arm (level of evidence: 1a-, degree of recommendation: D).

In the meta-analysis of Sgourakis *et al*[42], the corticosteroid-free immunosuppression group was equivalent to the steroid group in comparisons related to the following outcomes: acute rejection [mild: RR = 0.94 (0.69-1.29), P = 0.7] [moderate: RR = 1.02 (0.83-1.27), P = 0.8] [chronic rejection: RR = 1.52 (0.71-3.23), P = 0.2] and [steroid-resistant rejection: RR = 1.34 (0.87-2.08), P = 0.5]. Heterogeneity among studies in terms of acute rejection was observed. Considering overall acute rejection, in contrast to the results of meta-analysis (comparable results between treatment arms), metaregression showed that taking into account independently the RCT that replaced steroids [different regimens among studies *e.g.*, daclizumab (DAC), rabbit antithymocyte globulin, mycophenolate mofetil (MMF), or daclizumab and MMF], the outcome favored the corticosteroid-free immunosuppression arm [RR = 1.31 (1.09-1.58), P < 0.01)], while the reverse applied when steroids were not replaced. In the meta-analysis by Segev *et al*[41], the rates of rejection within the first three months were somewhat higher among the steroid-free arms [RR = 1.31 (1.04-1.64), P = 0.02) in studies where steroids were stopped, but not replaced. In contrast, the

Table 1	Evidence-based medicine levels of evidence[43]	
Level	**Therapy/prevention, etiology/harm**	**Prognosis**
1a	SR (with homogeneity[1]) of RCTs	SR (with homogeneity[1]) of inception cohort studies; CDR[2] validated in different populations
1b	Individual RCT (with narrow Confidence Interval[2])	Individual inception cohort study with > 80% follow-up; CDR[2] validated in a single population
1c	All or none[3]	All or none case-series
2a	SR (with homogeneity[1]) of cohort studies	SR (with homogeneity[1]) of either retrospective cohort studies or untreated control groups in RCTs
2b	Individual cohort study (including low quality RCT; *e.g.*, < 80% follow-up)	Retrospective cohort study or follow-up of untreated control patients in an RCT; Derivation of CDR[2] or validated on split-sample[4] only
2c	"Outcomes" Research; Ecological studies	"Outcomes" research
3a	SR (with homogeneity[1]) of case-control studies	
3b	Individual case-control study	

[1]A systematic review (SR) that is free of worrisome variations (heterogeneity) in the directions and degrees of results between individual studies. Studies displaying worrisome heterogeneity should be tagged with a "-" at the end of their designated level; [2]Clinical decision rule (CDR) (These are algorithms or scoring systems that lead to a prognostic estimation or a diagnostic category); [3]Met when all patients died before the Rx became available, but some now survive on it; or when some patients died before the Rx became available, but now none die on it; [4]Split-sample validation is achieved by collecting all the information in a single tranche, and subsequently artificially dividing this into "derivation" and "validation" samples. RCTs: Randomized control trials.

Table 2	Grades of recommendation[43]
A	Consistent level 1 studies
B	Consistent level 2 or 3 studies or extrapolations from level 1 studies
C	Level 4 studies or extrapolations from level 2 or 3 studies
D	Level 5 evidence or troubling inconsistent or inconclusive studies of any level

rates of rejection were markedly lower in the steroid-free arms [RR = 0.67 (0.48-0.96), $P = 0.03$) where steroids were replaced by other immunosuppressive agents. The rates of severe rejection also had a slightly higher trend, although not statistically significant, in studies where steroids were stopped, but not replaced [RR = 1.36 (0.63-2.93), $P = 0.4$]. In studies in which steroids were replaced by another IS agent, severe rejection [RR = 0.37 (0.20-0.68), $P = 0.001$] was markedly lower in the steroid-free arms.

The above-mentioned meta-analyses failed to provide a conclusive answer as many single studies resulted in a wide Confidence Interval and these meta-analyses had troublesome heterogeneity. Such evidence is inconclusive, and can thus only generate Grade D recommendations.

Four RCTs were published after these two meta-analyses[9,29,32,40].

Steroid replacement by daclizumab + MMF resulted in fewer cases of biopsy proven acute rejection (BPAR) at 24 wk in the corticosteroid-free immunosuppression arm (level of evidence: 1b, degree of recommendation: A).

The study by Otero *et al*[29] [Tacrolimus (TACRO) + ST (3 mo) *vs* TACRO + Daclizumab + MMF - steroids replaced] enrolled 77 patients per treatment group which was required to provide 80% power to detect a difference

between the null hypothesis (a rejection rate of 40% in both groups) and the alternative hypothesis (a rejection rate of 16% in the modified therapy group) with a 2-sided significance level of 0.05 to allow for an estimated 20% discontinuation rate. Significantly more patients in the standard therapy group experienced BPAR at 24 wk in comparison with patients in the modified (steroids replaced) therapy group (26.6% *vs* 11.5%, $P = 0.017$).

Initial steroid administration for two weeks and early Tacrolimus monotherapy is a feasible immunosuppression regimen without steroid replacement, although in view of chronic rejections, further investigations are needed (level of evidence: 1b, degree of recommendation: A).

In the study by Weiler *et al*[40] [TACRO + ST (steroids) for the first 2 wk followed by TACRO *vs* TACRO + ST (6 mo) - steroids not replaced], acute rejection after initiation of the study medication was comparable for both groups. Steroid-free immunosuppressive therapy leads to a higher rate of chronic rejection ($P = 0.023$). This study was statistically powered (the initial scheduled sample size of 50 per treatment group was based on an estimated difference in the incidence of steroid side-effects of 15% between the primary study endpoints).

Ab initio tacrolimus monotherapy is a viable immunosuppressive approach in liver transplantation and is associated with lower rejection rates compared to microemulsified cyclosporine (CyA) (level of evidence: 2b, degree of recommendation: B).

Monotherapy (microemulsified CyA/TACRO) in both groups was used in the study by Cholongitas *et al*[9]. Patients in the TACRO group, as compared to those in the CyA group, had a significantly lower number of mild ($P = 0.004$), severe ($P = 0.006$) and total ($P = 0.001$) rejection episodes per patient. Chronic rejection was observed in four (11%) patients receiving CyA. No patient receiving TACRO experienced chronic rejection ($P < 0.001$). This study was not statistically powered.

Adverse events: Renal insufficiency, *de novo* hypertension, neurological disorders and infectious complications do not differ significantly among steroid and steroid-free groups (level of evidence: 1a, degree of recommendation: B).

In a meta-analysis by Sgourakis *et al*[42], the corticosteroid-free immunosuppression group was equivalent to the steroid group in comparisons concerning the following outcomes: renal insufficiency [RR = 0.93 (0.78-1.11), $P = 0.4$] and severe renal insufficiency [odds ratio (OR) = 0.98 (0.52-1.81), $P = 0.9$] requiring hemofiltration, *de novo*

Table 3 Baseline study characteristics

Ref.	Indication	Recipients	Regimens	Outcomes	Study duration	Rejection treatments protocols
Belli *et al*[6] 2001	HCV positive	Group A = 13, Group B = 11, Group C = 13	RATG + AZA + CyA + ST (3 mo) /RATG + AZA + CyA /RATG + AZA + CyA + ribavirin	Acute rejection, chronic rejection, HCV recurrence	November 1997-November 1999	NS
Boillot *et al*[7] 2005	Adult patients undergoing first OLT	Group A = 351 (103), Group B = 347 (106)	TACRO + daclizumab/TACRO + ST (3 mo)	Acute rejection, corticosteroid resistant acute rejection, graft survival	July 2000-February 2002	Increasing TACRO dose and/or steroids
Eason *et al*[10] 2003	Adult patients undergoing first OLT	Group A = 59 (34), Group B = 60 (31)	RATG + TACRO + MMF/ST (3 mo) + TACRO + MMF	Patient survival, graft survival, rejection, adverse events, HCV recurrence	December 1999-August 2002	Increasing TACRO or adding MMF or sirolimus; steroids if no improvement after 48 h
Filipponi *et al*[11] 2004	HCV positive	Group A = 74, Group B = 66	Basiliximab + ST (3 mo) + CyA + AZA/ basiliximab + CyA + AZA	HCV recurrence, patient survival, graft survival, treatment failure	October 1998-March 2001	Methylprednisolone bolus for 3 d
Kato *et al*[14] 2007	HCV positive	1st Period Group A = 15, Group B = 16/2nd Period Group A = 16, Group B = 23	1st Period TACRO + daclizumab/TACRO + ST (3 mo)/2nd Period TACRO + daclizumab + MMF/TACRO + ST (3 mo) + MMF	Fibrosis stage, acute rejection, adverse events, predictors	November 1999-November 2001	Methylprednisolone bolus ± taper; OKT3 for severe or treatment-resistant rejection
Klintmalm *et al*[16] 2007 (updated by Klintmalm 2011)	HCV positive	Group A = 80, Group B = 79, Group C = 153	TACRO + ST (3 mo)/TACRO + ST (3 mo) + MMF/daclizumab + TACRO + MMF	Risk factors, rejection, HCV recurrence, treatment failure	NS	Methylprednisolone bolus ± taper; mild rejection increasing tacrolimus ± antimetabolite (MMF or azathioprine) Antilymphocyte antibody for corticosteroid-resistant rejection
Langrehr *et al*[18] 2002	HCV positive	Group A = 27, Group B = 26	TACRO + ST (3 mo)/TACRO + MMF	Rejection, HCV recurrence	NS	NS
Lerut *et al*[19] 2004 (updated by Lerut 2008)	Adult patients undergoing first OLT	Group A = 50, Group B = 50	TACRO + ST (3 mo)/TACRO	Acute rejection, graft survival, adverse events	NS	NS
Lladó *et al*[22] 2006 (updated by Llado 2008)	Adult patients undergoing first OLT	Group A = 102 (45), Group B = 96 (43)	Basiliximab + CyA + ST (3 mo)/ basiliximab + CyA	Acute rejection, patient survival, graft survival, infection	April 2001-September 2004	Methylprednisolone bolus for 3 d ± taper ± increase in TACRO
Lupo *et al*[17] 2005 (updated by Lupo 2008)	Adult patients undergoing first OLT	Group A = 20 (9), Group B = 21 (11)	CyA + ST (3 mo)/CyA + Basiliximab	Acute rejection	NS	Methylprednisolone bolus for 3 d
Margarit *et al*[25] 2005	Adult patients undergoing first OLT	Group A = 28 (20), Group B = 32 (15)	TACRO/TACRO + ST (3 mo)	Acute rejection, severe acute rejection, HCV recurrence, 3 yr-graft survival	October 1998-September 2000	Increasing tacrolimus dose; methylprednisolone bolus for 3 d ± taper for severe rejection
Moench *et al*[26] 2007 (updated by Weiler 2010)	Adult patients undergoing first OLT	Group A = 56 (15), Group B = 54 (16)	TACRO/TACRO + ST (6 mo)	Patient survival, graft survival, acute rejection, chronic rejection, adverse events	February 2000-August 2004	Methylprednisolone; tacrolimus adjusted higher level

Study	Population	Groups	Regimen	Outcomes	Study period	Steroid rescue/comments
Nashan et al[27] 2001	Adult patients undergoing first OLT	Group A = 25 (15); Group B = 26 (15)	Basiliximab + CyA + ST (3 mo)/Basiliximab + CyA + MMF OLT	Rejection, HCV recurrence	January 1999-December 2000	NS
Pageaux et al[30] 2004	Adult patients undergoing first OLT	Group A = 90; Group B = 84	Basiliximab + CyA + ST (6 mo)/basiliximab + CyA + placebo	Acute rejection, 6-mo graft and patient survival, treatment failure, recurrent HCV, adverse events	December 1999-August 2001	NS
Pelletier et al[31] 2005	Adult patients undergoing first OLT	Group A = 36; Group B = 36	TACRO + MMF + ST (3-6 mo)/TACRO + MMF	Rejection, HCV recurrence, graft survival patient survival	June 2002-May 2004	Pulse steroids
Reggiani et al[33] 2005	Adult patients undergoing first OLT	Group A = 18; Group B = 12	TACRO + MMF + ST (3 mo)/TACRO + MMF	Acute rejection, adverse events, pharmacokinetics of MPA	NS	NS/increasing tacrolimus for mild rejection; methylprednisolone bolus 3 d ± taper for moderate rejection; OKT3 for steroid-resistant rejection
Samonakis et al[34] 2006	HCV positive	Group A = 27; Group B = 29	TACRO/TACRO + ST (3-4 mo) + AZA	Acute rejection, survival, re-transplantation, adverse events	January 2000-January 2004	Methylprednisolone bolus for 3 d
Studenik et al[35] 2005	Adult patients undergoing first OLT	Group A = 19; Group B = 20	TACRO + daclizumab + ST (3 mo) + MMF/TACRO + daclizumab + MMF	Acute rejection	February 2003-November 2004	NS
Tisone et al[37] 1999	Adult patients undergoing first OLT	Group A = 22; Group B = 23	CyA + AZA + ST (3 mo)/CyA + AZA	Graft survival, adverse events, HCV recurrence	NS	Methylprednisolone bolus for 3 d only for severe rejection duct damage
Varo et al[38] 2005 (updated by Otero 2009)	Adult patients undergoing first OLT	Group A = 79; Group B = 78	TACRO + ST (3 mo)/TACRO + daclizumab + MMF	Acute rejection	NS	Up to 3 full courses of high dose steroids
Washburn et al[39] 2001	Adult patients undergoing first OLT	Group A = 15; Group B = 15	TACRO + MMF + ST (15 mo)/daclizumab + TACRO + MMF	Adverse events, rejection	April 1999-October 1999	Increasing tacrolimus dose; steroid bolus for moderate rejection
Manousou et al[24] 2009	HCV positive	Group A = 54; Group B = 49	TACRO/TACRO + AZA + ST (3 mo)	Progression to Ishak S4, graft failure resulting in retransplantation or patient death, immunological failure, patient survival, acute rejection, chronic rejection, steroid-resistant rejection, recurrent HCV	January 2000-June 2007	Pulse steroids
Ramirez et al[32] 2013	Adult patients undergoing first OLT	Group A = 20; Group B = 19	Basiliximab + TACRO + EC-MPS + ST (6 mo)/basiliximab + TACRO + EC-MPS	Patient survival, graft survival, rejection, adverse events	February 2006-November 2007	NS
Neumann et al[28] 2012	HCV positive	Group A = 67; Group B = 68	TACRO + ST (3 mo)/TACRO + DAC	Viral load of HCV at 12 mo, the incidence of BPAR, patient and graft survival at 12 mo, renal function, adverse events	June 2005-Jun 2008	Increasing tacrolimus dose to trough levels of 15 ng/mL ± pulses of corticosteroids up to 1000 mg/d for 3 consecutive days
Takada et al[36] 2013	Living donor liver transplantation HCV positive	Group A = 35; Group B = 40	TACRO + ST (3 mo)/TACRO + MMF	Event-free survival: histological recurrence of hepatitis C, BPAR resistant to 2 sets of steroid pulse therapy, hepatocellular carcinoma recurrence, Re-transplantation, Patient death	NS	Pulse steroids
Lerut et al[19] 2008	Adult patients undergoing first OLT	Group A = 78; Group B = 78	TACRO + ST (64 d)/TACRO	Graft and patient survival, incidences of TAC monotherapy and of low-dosage TAC monotherapy, renal insufficiency, diabetes mellitus, hypercholesterolemia, hyperuricemia, arterial hypertension, infectious, tumor complications, and performance status	February 2000-September 2004	Corticosteroid-sensitive rejection was treated with 3 to 5 oral or IV boluses of 200-mg Methylprednisolone. CRC was treated with a 10-d IV course of muromonab orthoclone OKT3

Ref	Population	Groups (n)	Treatment	Outcomes	Study period	Rejection treatment
Becker et al[5] 2008	Adult patients undergoing first OLT or split liver allograft transplantation	Group A = 305, Group B = 297	TACRO + daclizumab/TACRO + MMF	Rejection, overall survival and allograft survival, renal function	March 2005-June 2007	Acute rejection was treated with three 1 g/d methylprednisolone
Cholongitas et al[9] 2011	Chronic liver disease	Group A = 36, Group B = 30	CyA/TACRO	Death	January 1996-January 1997	NS
Gerhardt et al[12] 2009	Adult patients undergoing first OLT	Group A = 8, Group B = 13	CNI/MMF vs a MMF/prednisone	Renal function	May 2003-May 2005	"mild" rejection episodes were treated with steroid boluses. Steroid pulse therapy
Klintmalm et al[15] 2011	HCV positive	Group A = 77, Group B = 72, Group C = 146	TACRO + ST (3 mo)/TACRO + ST (3 mo) + MMF/daclizumab + TACRO + MMF	Acute rejection, HCV recurrence, survival	NS	ACR was treated with an increase in TACRO to 15 ng/mL without a corticosteroid bolus and recycle. Moderate to severe ACR (ACR 4) was treated with a 1.0-g bolus of methylprednisolone, followed by a 6-d steroid taper of intravenous methylprednisolone or oral prednisone
Lladó et al[21] 2008	HCV positive	Group A = 46, Group B = 43	Basiliximab + CyA + ST (3 mo)/ basiliximab + CyA	Acute rejection, patient and graft survival, adverse events (infections and metabolic decompensations) HCV recurrence	April 2001-September 2004	Methylprednisolone bolus for 3 d ± taper ± increase in TACRO
Lupo et al[23] 2008	Adult patients undergoing first OLT	Group A = 21, Group B = 26	CyA + ST (3 mo)/CyA + Basiliximab	Acute rejection, patient and graft survival, HCV recurrence, medical and surgical complications, infections	November 2002-November 2005	Methylprednisolone bolus for 3 d
Otero et al[29] 2009	Adult patients undergoing first OLT	Group A = 79, Group B = 78	TACRO + ST (3 mo)/TACRO + Daclizumab + MMF	Acute rejection, time to rejection, patient and graft survival, HCV status, hepatic and renal function	May 2002-December 2003	Up to 2 courses of high dose steroids for 3 d. Corticosteroid-resistant rejection episode was treated with anti-lymphocyte therapy
Weiler et al[40] 2010	Adult patients undergoing first OLT	Group A = 56, Group B = 54	All patients TACRO + steroids for the first 2 wk TACRO/TACRO + ST (6 mo)	Patient survival, organ survival, steroid side-effects, acute rejection, chronic rejection, HCV recurrence	February 2000-August 2004	Methylprednisolone; tacrolimus adjusted higher level
Junge et al[13] 2005	Recipients with autoimmune hepatitis	Group A = 14, Group B = 16	TACRO + steroids/TACRO + MMF	Graft and patient survival, acute rejection, liver functions, glucose metabolism, bone density, blood pressure, renal function, drug-related side-effects, infections	NS	Mild or moderate rejection: methylprednisolone pulse therapy severe rejection: high-dose steroids + monoclonal antibody
Bonaccorsi-Riani et al[8] 2012	HCV positive	Group A = 14, Group B = 21	TACRO + steroids (2 mo)/TACRO + placebo	1 and 5 yr survival; HCV recurrence, retransplantation, death	NS	NS

Numbers within brackets in the third column show the number of hepatitis C virus (HCV) transplanted patients; CyA: Cyclosporine; TACRO: Tacrolimus; ST: Steroids; RATG: Rabbit antithymocyte globulin; AZA: Azathioprine; MMF: Mycophenolate mofetil; OKT3: Murine monoclonal IgG2a antibody; EC-MPS: Enteric-coated mycophenolate sodium; BPAR: Biopsy-proven acute rejection; S4: Stage 4; CRC: Corticosteroid-resistant rejection; OLT: Orthotopic liver transplantation; BPAR: Biopsy proven acute rejection; NS: Non-significance.

Table 4 Summary of the strength and quality of the evidence

Intervention	Level of evidence	Degree of recommendation
Studies including adult patients undergoing first OLT for any indication		
Steroid replacement results in fewer cases of overall acute rejection in the corticosteroid-free immunosuppression arm	1a-	D
Steroid replacement by daclizumab + MMF results in fewer cases of BPAR at 24 wk in the corticosteroid-free immunosuppression arm	1b	A
Initial steroid administration for two weeks and early tacrolimus monotherapy is a feasible immunosuppression regimen without steroid replacement, although in view of chronic rejections, further investigations are needed	1b	A
Ab initio tacrolimus monotherapy is a viable immunosuppressive approach in liver transplantation and is associated with lower rejection rates compared to microemulsified cyclosporine	2b	B
Renal insufficiency, de novo hypertension, neurological disorders and infectious complications do not differ significantly among steroid and steroid-free groups	1a	B
Diabetes mellitus, cholesterol levels and CMV infection had a higher incidence in the steroid group. The differences in cases of diabetes mellitus and hypercholesterolemia are independent of steroid replacement	1a-	D
Hypertension, thrombocytopenia, renal impairment and overall incidence of infections do not differ significantly among steroid and steroid-free groups (steroids replaced by daclizumab + MMF)	1b	A
Early tapering down of steroids to tacrolimus monotherapy is possible with significantly fewer cases of diabetes and hypercholesterolemia	1b	A
Side-effects related to monotherapy with microemulsified cyclosporine or tacrolimus are comparable	2b	B
Complete corticosteroid avoidance in adult OLT using basiliximab induction with CNI and EC-MPS maintenance is as safe and as effective as standard corticosteroid containing immunosuppression	2b	B
No significant differences were noted between treatment groups in terms of patient and graft survival regardless of steroid replacement	1b	A
Actuarial 5-yr patient and graft survival related to monotherapy with microemulsified cyclosporine or tacrolimus are comparable	2b	B
Steroid withdrawal should be attempted in OLT recipients with underlying autoimmune hepatitis	2b-	D
Which immunosuppression regimen? Both, tacrolimus-based regimens with daclizumab induction or the addition of MMF, allow for avoidance of steroid treatment	1b	A
Studies addressing exclusively transplanted HCV patients		
A significant reduction in HCV recurrence independent of steroid replacement may be expected in steroid-free groups	1a-	D
MMF does not appear to have a significant antiviral effect despite early reports	1b	A
Male gender of donors and recipients, living donors, cold ischemia times, acute rejection, and early histological recurrence are related to the development of advanced hepatitis	1b	A
Donor age, grade 2 inflammation at day 90 or one-year liver biopsy and diagnosis of acute hepatitis may be associated with the development of bridging fibrosis or cirrhosis	2b	B

CMV: Cytomegalovirus; MMF: Mycophenolate Mofetil; OLT: Orthotopic liver transplantation; EC-MPS: Enteric-coated mycophenolate sodium; BPAR: Biopsy-proven acute rejection; CNI: Calcineurin inhibitor; HCV: Hepatitis C virus.

hypertension development [RR = 1.07 (0.9-1.27), $P = 0.4$], neurological disorders [OR = 0.76 (0.51-1.13), $P = 0.2$] and infectious complications [RR = 1.07 (0.96-1.2), $P = 0.2$].

In a meta-analysis by Segev *et al*[41], the corticosteroid free-immunosuppression group was equivalent to the steroid group in comparisons related to the following outcomes: cumulative risk of hypertension [RR = 0.84 (0.69-1.02), $P = 0.08$] and infection [RR = 0.97 (0.88-1.08), $P = 0.6$].

Degree of recommendation "B" was extrapolated because data used in the included studies were clinically different despite the fact that there was no heterogeneity or wide Confidence Intervals among outcomes in both meta-analyses.

Diabetes mellitus, cholesterol levels and cytomegalovirus (CMV) infection showed a higher incidence in the steroid group. The differences in cases of diabetes mellitus and hypercholesterolemia were independent of steroid replacement (level of evidence: 1a-, degree of recommendation: D).

The development of post-transplant diabetes mellitus [RR = 1.86 (1.43-2.41), $P < 0.001$], cholesterol levels at 6 mo [weighted mean difference (WMD) = 19.71 (13.7-25.7), $P < 0.001$] and CMV infection [RR = 1.47 (0.99-2.17), $P < 0.05$] favored the corticosteroid-free immunosuppression group as reported by Sgourakis *et al*[42].

Similar results were published in the meta-analysis by Segev *et al*[41]: Significant reductions in cholesterol [standard mean difference = -0.41 [(-0.62)-0.20], $P < 0.001$] and the risk of CMV infection [RR = 0.52 (0.35-0.76), $P = 0.001$], were observed in the steroid-free groups.

Both meta-analyses had statistically significant heterogeneity of studies, especially in CMV infection and some studies had wide Confidence Intervals.

Metaregression analysis in the former[42] disclosed that there was no difference between studies that replaced or did not replace steroids in the corticosteroid free-immunosuppression group ($P = 0.087$). The latter[41] also supports that the risk of diabetes [RR = 0.29 (0.18-0.47), $P < 0.001$], was markedly lower in the steroid-free arms.

Four RCTs were published after these two meta-

analyses[9,29,32,40].

Hypertension, thrombocytopenia, renal impairment and overall incidence of infections do not differ significantly among steroid and steroid-free groups (steroids replaced by daclizumab + MMF) (level of evidence: 1b, degree of recommendation: A).

In the sufficiently powered study by Otero *et al*[29] [TACRO + ST (3 mo) *vs* TACRO + daclizumab + MMF - steroids replaced], although more patients in the standard therapy group reported hypertension (26.6% *vs* 20.5%), thrombocytopenia (15.2% *vs* 12.8%), new-onset diabetes mellitus (13.9% *vs* 9.0%), and renal impairment (27.8% *vs* 19.2%), these differences were not statistically significant. Overall infections occurred in 19.2% of the modified therapy group *vs* 11.4% of the standard therapy group (*P* = 0.172).

Early tapering down of steroids to a tacrolimus monotherapy is possible with significantly fewer cases of diabetes and hypercholesterolemia (level of evidence: 1b, degree of recommendation: A).

In the sufficiently powered study by Weiler *et al*[40] [all patients TACRO + steroids for the first 2 wk followed by TACRO/TACRO + ST (6 mo) - steroids not replaced], statistically significant differences in diabetes (53% in the steroid group *vs* 30%, *P* = 0.024) and hypercholesterolemia (41% in the steroid group *vs* 10%, *P* = 0.002) were demonstrated at six months. A statistical difference in the osteoporosis rate was insignificant.

Side-effects related to monotherapy by microemulsified cyclosporine or tacrolimus are comparable (level of evidence: 2b, degree of recommendation: B).

Monotherapy (microemulsified CyA/TACRO) in both groups was used in the study by Cholangitas *et al*[9]. Twenty-eight (77%) patients in the CyA group developed renal dysfunction (defined as GFR < 60 mL/min) at least once, compared to 45 (36%) in the TACRO group (*P* < 0.001), although this difference remained at the margin of significance at five years. Side-effects related to immunosuppression were similar between the two groups at one, two and five years after liver transplantation.

Complete corticosteroid avoidance in adult OLT using basiliximab induction with calcineurin inhibitor and enteric-coated mycophenolate sodium (EC-MPS) maintenance is as safe and as effective as standard corticosteroid containing immunosuppression (level of evidence: 2b, degree of recommendation: B).

In the study by Ramirez *et al*[32] [Basiliximab + TACRO + EC-MPS + ST (6 mo) *vs* basiliximab + TACRO + EC-MPS], mean cholesterol levels were similar in both groups from baseline to 12 mo post-OLT. Mean arterial pressure levels were significantly higher in the corticosteroid group as opposed to the corticosteroid-free group at three and 12 mo post-OLT.

Graft and patient survival: No significant differences were noted between treatment groups in terms of patient and graft survival regardless of steroid replacement (level of evidence: 1b, degree of recommendation: A).

In the meta-analysis by Sgourakis *et al*[42], relevant

comparisons were equivalent among the corticosteroid-free immunosuppression group *vs* the steroid group in terms of the following outcomes: overall number of deaths during follow-up [RR = 0.9 (0.72-1.13), *P* = 0.36], one-year patient survival [OR = 0.1 (0.69-1.45), *P* = 0.9], one-year graft survival [OR = 0.8 (0.56-1.15), *P* = 0.2], retransplantation [OR = 0.82 (0.45-1.52), *P* = 0.6], deaths up to 6 mo [RD = -0.01 (-0.04-0.02), *P* = 0.5] and 3-mo graft survival [OR = 1.24 (0.79-1.25), *P* = 0.4). Only 7 studies[7,9,10,26,28-30] gave detailed information on the percentage of patients in each treatment arm which had received the allocated regimen. The corticosteroid-free immunosuppression group was superior in terms of the number of patients receiving the allocated intervention [OR = 1.55 (1.17-2.05), *P* = 0.003].

No differences between steroid-free and steroid-based protocols were observed in terms of death [RR = 0.95 (0.73-1.24), *P* = 0.7] and graft loss [RR = 0.95 (0.76-1.19), *P* = 0.6] in the meta-analysis by Segev *et al*[41].

Both meta-analyses included some studies with wide Confidence Intervals, while data in the latter meta-analysis have not been extrapolated to the specific time period.

In the sufficiently powered study by Otero *et al*[29] (steroids replaced) no significant differences emerged between treatment groups in terms of patient and graft survival; however, the time to rejection for patients in the standard therapy group was significantly shorter than that noted in the modified therapy group (*P* = 0.044).

In the sufficiently powered study by Weiler *et al*[40] (steroids not replaced), patient (*P* = 0.236) and graft survival (*P* = 0.509) was similar in both groups. In total, eight patients (7.3%) were retransplanted within 5 years, four (7.1%) from the placebo group, and four (7.4%) from the steroid group.

In the study by Ramirez *et al*[32], no significant differences in patient and death-censored graft survival rates between the two groups (corticosteroids and corticosteroid-free) were observed. The 1-, 3-, and 5-year patient survival were as follows: 100% *vs* 95%, 85% *vs* 63%, and 80% *vs* 63%. The 1-, 3-, and 5-year graft survival rates in the corticosteroid and corticosteroid-free groups were as follows: 100% *vs* 95%, 85% *vs* 63%, and 75% *vs* 63%, respectively.

Actuarial 5-year patient and graft survival related to monotherapy with microemulsified cyclosporine or tacrolimus are comparable (level of evidence: 2b, degree of recommendation: B).

Monotherapy in both groups was used in the study by Cholangitas *et al*[9]. Actuarial survival according to Kaplan-Meier curves at five years was 72% for TACRO and 70% for CyA. Graft survival at five years was 59% for TACRO and 57% CyA. Neither patient survival nor graft survival differed statistically between the groups. Only two patients in the TACRO group required a second LT, compared to five (14%) in the CyA group (*P* = 0.007).

Liver transplant recipients with autoimmune hepatitis: Steroid withdrawal should be attempted in OLT recipients with underlying autoimmune hepatitis (level of

evidence: 2b-, degree of recommendation: D).

Only one RCT[13] exclusively analyzed patients with autoimmune hepatitis (AIH). The 2-year survival in the prednisone group was 93% *vs* 100% in the steroid-free group who received MMF. No differences were observed with regard to graft function, acute rejection, renal function, and infectious complications. The prednisone group showed significantly elevated glucose levels with higher HbA1c and insulin requirements. The mean serum cholesterol level was significantly lower and bone density showed significant improvement in the MMF as opposed to the prednisone group (both outcomes: *P* < 0.01). The authors suggested that steroid withdrawal should be attempted in OLT recipients with underlying AIH. This is a low sample size study, where the randomization procedure and patient allocation were not disclosed.

Which steroid-free regimen? Both, TACRO-based regimens with DAC induction or the addition of MMF, allow for avoidance of steroid treatment (level of evidence: 1b, degree of recommendation: A).

In the large sufficiently powered, multicentre, randomized, open-label, parallel group, phase III trial conducted by Becker *et al*[5], 602 patients were enrolled and randomized to treatment: 305 patients were randomized to the TACRO/DAC group and 297 to the TACRO/MMF group. Approximately 70% of patients in each group completed the study. The overall estimated rate of patients free of BPAR that required treatment within 3 mo of transplantation was 81.5% in the TACRO/DAC group and 82.2% in the TACRO/MMF group. Differences were found in the incidence of causally related adverse events which was significantly lower in the TACRO/DAC group than that in the TACRO/MMF group: 76.1% and 82.8%, respectively (*P* < 0.05). Conversely, renal disorders were more often reported as an adverse event in the TACRO/DAC group than in the TACRO/MMF group. The authors concluded that TAC monotherapy after DAC induction was associated with significantly less leucopenia and bacterial infection. Both TACRO-based regimens, DAC induction or dual therapy with MMF, allow for avoidance of steroid treatment, thereby eliminating risks associated with steroids, while providing satisfactory levels of immunosuppression.

Results of studies addressing exclusively transplanted HCV patients

HCV recurrence: A significant reduction in HCV recurrence independent of steroid replacement may be expected in the steroid-free groups (level of evidence: 1a-, degree of recommendation: D).

In the meta-analysis by Sgourakis *et al*[42], the corticosteroid-free immunosuppression group was equivalent to the steroid group in comparisons pertaining to the following outcomes: overall deaths in HCV patients [RR = 0.92 (0.52-1.65), *P* = 0.8], deaths in HCV-recurrence patients {RD = 0.01 [(-0.05)-0.07], *P* = 0.7}, one-year patients [OR = 0.63 (0.37-1.08), *P* = 0.1] and one-year

graft survival [OR = 0.68 (0.42-1.08), *P* = 0.08]. The corticosteroid-free immunosuppression group was superior in terms of the relative risk of HCV recurrence [RR = 1.15 (1.01-1.13), *P* < 0.05], acute graft hepatitis [OR = 3.15 (1.18-8.40), *P* = 0.03] and the number of patients failing treatment: collectively, patients with graft loss/deaths/withdrawal [OR = 1.87 (1.33-2.63), *P* = 0.0001]. Metaregression analysis also disclosed that there was no difference between studies that replaced or did not replace steroids in the corticosteroid-free immunosuppression group in terms of HCV recurrence (*P* = 0.610).

Significant reductions in HCV recurrence [RR = 0.90 (0.82-0.99), *P* = 0.03] were observed in the steroid-free groups in the meta-analysis by Segev *et al*[41].

The fact that both the above-mentioned meta-analyses included studies with less than 6 mo of follow-up and that HCV recurrence is defined in many different ways among studies (Ishak score, fibrosis, HCV RNA *etc*.) must be taken into consideration.

Eight RCTs exclusively addressed HCV transplanted patients after the publication of the two meta-analyses[9,15,24,28,29,32,36,40], three of which involved deceased donors[15,24,28], one a living donor[36] and the remaining four all etiologies of deceased donor transplantation[9,29,32,40].

In the sufficiently powered study by Neumann *et al*[28], patients who had received antiviral treatment during the study were excluded. The percentage of patients free of HCV recurrence at 12 mo was 19.1% for the TACRO/DAC steroid-free protocol and 13.8% for the TACRO/ST protocol, with a significant difference in survival curves between treatments (*P* = 0.020). HCV recurrence censored for antiviral treatment favored the TACRO/DAC immunosuppression protocol at a rate of 20.2% *vs* 13.1% in the TACRO/ST group (*P* = 0.022). The overall estimated rate of patient survival was significantly lower in the TACRO/DAC arm (*P* = 0.025). The estimated rate of graft survival was numerically lower in the TACRO/DAC arm. The rate of graft loss was 19.4% in the TACRO/DAC arm and 8.8% in the TACRO/ST arm. The overall frequency of BPAR was significantly lower in the TACRO/DAC than in the TACRO/ST arm (*P* = 0.048).

Although there was a tendency for later HCV recurrence and a lower incidence of rejection, the authors also observed a higher dropout rate and a lower patient survival rate with TACRO/DAC compared to the TACRO/ST arm and concluded that it is difficult to recommend a steroid-free protocol for HCV-positive patients due to the afore-mentioned study limitations.

In the study by Manousou *et al*[24], antiviral treatment for HCV recurrence [after Ishak stage 4 was reached] was used in six out of 54 monotherapy (MT) and eight out of 49 triple therapy (TT) patients, with three in each group achieving sustained virological response. Overall mortality was not significantly different between the groups. The difference in reaching the primary endpoint (Stage 4 fibrosis) was significantly (*P* = 0.045) in favor of triple therapy patients. Rejection episodes assessed by protocol biopsies that were histologically proven and/or required

methylprednisolone (30% *vs* 49%) were less frequent in the MT group. Retransplantation rates (7.8% for TT and 9.6% for MT) and chronic rejection rates (2% for TT and 3.8% for MT) were not different. This randomized trial supported the benefit of low-dose and slowly tapered steroids as well as azathioprine after liver transplantation for HCV-positive recipients.

MMF does not appear to have a significant antiviral effect despite early reports (level of evidence: 1b, degree of recommendation: A).

In the sufficiently powered study by Klintmalm *et al*[15], clinically significant HCV in the three arms [TACRO + ST (3 mo)/TACRO + ST (3 mo) + MMF/daclizumab + TACRO + MMF] occurred in 69.5%, 75.9%, and 68.1% within 2 years. None of these differences was statistically significant. The 1- and 2-year patient and graft survival rates in the three arms were similar. The 2-year graft survival rates were 79.1%, 79.8%, and 85.1%, respectively (no significant differences). By day 730, clinically significant acute rejection had occurred in 14.3%, 12.5%, and 13.7% of the patients in the three arms. None of the differences among the groups was significant. The authors found no evidence that MMF influenced HCV progression.

With regard to living donor liver transplantation in the study by Takada *et al*[36], antiviral treatment with interferon and ribavirin was considered for HCV recurrence. A sustained virological response was achieved in 44.4% of patients in the ST group and in 66.7% of patients in the MMF group ($P = 0.16$). The 1-, 3-, and 5-year overall survival rates were 94.1%, 87.6%, and 82.7%, respectively, for the ST group and 92.5%, 84.5%, and 81.0%, respectively, for the MMF group ($P = 0.28$). BPAR requiring treatment with an ST bolus injection occurred in four patients from the ST group and in 13 patients from the MMF group ($P = 0.051$).

Predictors of the development of advanced hepatitis: Male gender of donors and recipients, living donors, cold ischemia times, acute rejection, and early histological recurrence are related to the development of advanced hepatitis (level of evidence: 1b, degree of recommendation: A).

In the sufficiently powered study by Klintmalm *et al*[15], Cox Regression showed that male gender of donors and recipients, living donors, cold ischemia times, acute rejection, and early histological recurrence (grade 2 inflammation on the 90-d liver biopsy sample or stage 1 fibrosis at one-year biopsy) were related to the development of advanced hepatitis.

Predictors of advanced fibrosis: Donor age, grade 2 inflammation at day 90 or one-year liver biopsy and diagnosis of acute hepatitis may be associated with the development of bridging fibrosis or cirrhosis (level of evidence: 2b, degree of recommendation: B).

According to a multivariate Cox analysis, the donor age and grade 2 inflammation at day 90 or one-year liver biopsy were associated with the development of bridging fibrosis or cirrhosis; the administration of murine mono-

clonal IgG2a antibody or thymoglobulin approached significance[15]. In yet another study, two independent predictors of fibrosis stage $\geq$ 4 were significant: randomization to monotherapy [OR = 0.7 (0.066-0.847)] and diagnosis of acute hepatitis [OR = 3.59 (1.108-9.823)][24].

However, the former study[15] observed subjects for only 2 years after transplantation, and some patients refused liver biopsy in the second year. Thus, the authors could not dismiss the possibility that differences might have been observed if all subjects had been biopsied or the follow-up had been longer. The latter study[24] was not sufficiently powered.

CONCLUSION

Considering adult patients undergoing first OLT

It seems that steroid replacement results in fewer cases of overall acute rejection in the corticosteroid-free immunosuppression arm, although the evidence is of moderate quality. Steroid replacement by daclizumab plus MMF or early tacrolimus monotherapy after initial steroid administration for two weeks, are strong alternatives. In terms of patient and graft survival and regardless of steroid replacement, steroid-free immunosuppression is strongly recommended.

Adverse events such as renal insufficiency, *de novo* hypertension, neurological disorders and infectious complications did not differ significantly among steroid and steroid-free groups. Diabetes mellitus, cholesterol levels and CMV infection showed a higher incidence in the steroid group. The differences in cases of diabetes mellitus and hypercholesterolemia were independent of steroid replacement.

Considering transplanted HCV patients

MMF does not appear to have a significant antiviral effect despite early reports. Male gender of donors and recipients, living donors, cold ischemia times, acute rejection, and early histological recurrence are related to the development of advanced hepatitis.

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TOPIC HIGHLIGHT

WJG 20ᵗʰ Anniversary Special Issues (7): Liver transplant

Clinical mycophenolic acid monitoring in liver transplant recipients

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Abstract

In liver transplantation, the efficacy of mycophenolate mofetil (MMF) has been confirmed in clinical trials and studies. However, therapeutic drug monitoring for mycophenolic acid (MPA) has not been fully accepted in liver transplantation as no long-term prospective study of concentration controlled *vs* fixed-dose prescribing of MMF has been done. This review addressed MPA measurement, pharmacokinetic variability and reasons of this variation, exposure related to acute rejection and MMF-associated side effects in liver transplant recipients. Limited sampling strategies to predict MPA area under the concentration-time curve have also been described, and the value of clinical use needs to be investigated in future. The published data suggested that a fixed-dosage MMF regimen might not be suitable and monitoring of MPA exposure seems helpful in various clinical settings of liver transplantation.

Key words: Mycophenolate mofetil; Mycophenolic acid; Pharmacokinetics; Therapeutic drug monitoring; Liver transplantation

Core tip: We discussed the methods of mycophenolic acid (MPA) monitoring, pharmacokinetic characteristics, clinical exposure related to acute rejection and mycophenolate mofetil (MMF) associated side effects in liver transplant recipients. We also introduced the methods of limited sampling strategies to predict the MPA area under the concentration-time curve. It demonstrated that a fixed-dosage MMF regimen might not be suitable. In clinical settings, monitoring of MPA exposure seems reasonable and necessary.

INTRODUCTION

Mycophenolate mofetil (MMF, CellCept, Hoffman-La Roche) has almost full bioavailability by oral intake and is a pro-drug that is hydrolyzed to release mycophenolic acid (MPA)[1]. Subsequently MPA is metabolized to a major phenolic glucuronide, mycophenolic acid glucuronide (MPAG), and a minor acyl glucuronide (AcMPAG)[2-4]. MPA, the active compound of MMF, is a selective, reversible and non-competitive inhibitor of inosine monophosphate dehydrogenase in process of de novo purine synthesis in T and B lymphocytes[5]. As a result nucleic acid synthesis is arrested and immune reaction to allograft is inhibited.

As a major immunosuppressive agent, MPA has been widely used for the prevention of acute rejection in transplant recipients[6]. A dose of 1-1.5 g (fixed-dose) adminis-

tered orally or intravenously twice a day is recommended for use in renal, cardiac and liver transplant patients in the product leaflet of Hoffman-La Roche Ltd[7]. However, wide inter-patient variability in MPA exposure has been showed in renal, heart and liver transplant patients on a fixed MMF dose[1,8,9]. It is confirmed in renal transplantation that compared with fixed-dose regimen, MPA concentration controlled regimen can reduce the risk of treatment failure and acute rejection in recipients 12 mo post-transplant with no increase in adverse events[10]. Individualizing MMF dose instead of using a fixed dose might be helpful to optimize immunosuppression and minimize potential toxic effects. Carrying out therapeutic drug monitoring (TDM) seems reasonable and necessary and routine monitoring for MPA is increasingly performed. However, the experience with TDM for MPA in liver transplantation is much limited compared to lots of investigations performed in kidney transplant patients. At present, a fixed dose of 1 or 1.5 g twice daily of MMF is the standard protocol in liver transplantation with adjustments only in relation to side effects or to its efficacy[11]. No more MPA monitoring-based guidelines for MMF dosage have been set up[12]. It is necessary to study the MPA pharmacokinetics and to carry out TDM of MMF in liver transplant recipients.

In this review, we will focus on five areas in liver transplant recipients: (1) MPA efficacy and MMF-related side effects; (2) methods for measuring MPA concentration; (3) MPA pharmacokinetics; (4) limited sampling strategy (LSS); and (5) MPA concentration-effect relationship.

MPA EFFICACY AND MMF-RELATED SIDE EFFECTS IN LIVR TRANSPLANATION

MMF has been successfully used with a reduced dosage of calcineurin inhibitor (CNI) and steroids to reduce the rate of acute rejection, lessen side effects of CNI after liver transplantation and improve long-term survival rates of allografts and recipients[13-15]. In a randomized double-blind comparative study of MMF and azathioprine in primary liver transplant recipients, the incidence of acute rejection or graft loss was 47.7% in the azathioprine-treated patients and 38.5% in the MMF-treated patients during the first 6 mo after transplantation[16]. Recently, Goralczyk *et al*[17] reported the results of a systematic review and meta-analysis of randomized controlled trials of CNI sparing with MMF in liver transplantation. The authors obtained the conclusion that *de novo* use of MMF in combination with low-dose tacrolimus (TAC) is not associated with an increased risk of acute rejection, graft loss, or death and has an acceptable side effect profile. Ringe *et al*[18] reported that use of TAC plus MMF immunosuppressive regimen without corticosteroids from the beginning after liver transplantation led to a graft survival rate of 83.9 % at 2 years.

MMF has no nephrotoxity and no effect on the lipid profile or other cardiovascular risk factors such as sys-temic hypertension or diabetes mellitus[19]. MMF has been widely used to improve the renal function commonly associated with CNI[20,21]. Its nephroprotective effect and promotion of allograft tolerance after liver transplantation were confirmed with replaced CNI or reduced or interrupted CNI therapy in three randomized controlled trials[22-24]. Recently, Kriss *et al*[25] reported that serum creatinine and calculated glomerular filtration rate (GFR) improved in 23 cases on MMF monotherapy compared with 23 recipients remaining on CNI-based therapy. Improvement was significantly pronounced in patients with milder renal dysfunction with a decrease in serum creatinine (1.63 ± 0.29 mg/dL *vs* 1.34 ± 0.26 mg/dL, $P = 0.02$) at last follow-up. In a retrospective analysis of pediatric liver transplantation by Evans *et al*[26], there was a statistically significant increase to a median calculated GFR of 69 (28-114) mL/min per 1.73 m^2 by 1 mo and a further increase to a median calculated GFR of 77 (24-105) mL/min per 1.73 m^2 by 2 mo with MMF monotherapy or low-dose cyclosporine A (CsA) or TAC, after which time calculated GFR was maintained. MMF treatment provided safe and effective immunosuppression and allowed CsA or TAC to be discontinued or reduced, leading to improvement of renal function.

CNI increased cardiovascular risk after liver transplantation. Aberg *et al*[27] analyzed the cardiovascular risk of 77 recipients based on CNI and antibodies at 5 years after liver transplantation. At least one cardiovascular risk factor developed in 92% of patients, and the prevalence of treated hypertension, dyslipidemia, overweight, obesity and diabetes were 71%, 61%, 32%, 13% and 10%, respectively. Antibody therapy was associated with a 1.49-fold increase in the risk of hypertension (95%CI: 1.15-1.94) and a 6.43-fold increase in the risk of diabetes. In a randomized prospective study by Junge *et al*[28], TAC with MMF compared TAC with corticosteroid significantly decreased glucose levels with lower HbA1c and the need for insulin as well as significantly reduced serum cholesterol and the incidence of osteopenia. It was confirmed in some studies that immunosuppressive protocol based on reduced doses of TAC[22,29] or corticosteroids[30] with MMF could improve blood pressure with reduction of antihypertensive medication.

In summary, the protocol using MMF with reduced TAC improves renal function, decreases the cardiovascular risk and avoids steroid-associated adverse effects.

The principal complications of MMF are gastrointertinal effects (nausea, vomiting, abdominal pain and diarrhea) and myelosuppression (leucopenia, anaemia and thrombocytopenia)[19]. In a study by Hao *et al*[31], 66.7 % of the patients had at least one episode of MMF-related side effects of hematologic disorder (36.51%), gastrointestinal reaction (25.40%) and infection (20.63%) during the study evaluation up to the third post-transplantation month. For 34 of the patients (53.97%), the symptoms disappeared until MMF was decreased gradually in dosage or stopped. Tredger *et al*[32] reported that a total of 96 adverse events possibly associated with MMF therapy

were well documented in the 147 adult patients, mainly including gastrointestinal dysfunction, leucopenia and infection.

In the study by Wiesner *et al*[16], diarrhea occurred in 51.3% of liver transplant recipients receiving MMF (1.5 g, twice daily) and corticosteroids. It seems that CNI therapy with MMF is associated with a higher incidence of diarrhea than monotherapy with MMF in liver transplantation. Diarrhea was observed in 31.4% of cases using MMF combined with CNIs[33]. For mono-therapy with MMF, a lower rate of diarrhea (14%-15%) was showed[34-36]. In stable renal transplant recipients, Maes *et al*[37] reported that gastric emptying of solids was significantly faster in patients treated with TAC compared with those with CsA. Cantarovich *et al*[13] reported that the incidence of diarrhea was 18% in liver transplantation patients using cyclosporine and MMF regimen, while the incidence of diarrhea was 38.63% in patients using MMF combined with TAC in a study by Xia *et al*[38].

METHODS FOR MEASURING MPA CONCENTRATION

Methods used for measurement of MPA concentration should be sensitive, accurate, specific, rapid, convenient and economical. Different methods were developed to determine total or unbound MPA (free MPA, fMPA) and MPA metabolites. These methods can be classified as chromatographic methods and immunoassays.

Chromatographic methods

Chromatographic methods have the advantages of good specificity and sensitivity. They are especially useful in monitoring the MPA and its metabolites simultaneously. However, these methods have the common shortcomings including complex sample preparation, which is labor-intensive and time-consuming. Chromatographic methods are suitable for laboratories with large sample load. Based on the variance in the detective method, chromatographic based assays used for MPA monitoring can be classified as high-performance liquid chromatography (HPLC) with ultraviolet (UV) or fluorescence detector and LC-MS/MS assay.

Determination of total MPA

Although LC-MS/MS is the most sensitive assay, HPLC-UV is sufficient in the monitoring of total MPA. Different UV absorption wavelengths were selected for MPA monitoring[39-41]. Most of these assays had the lower limit of quantification (LLOQ) of about 0.2 μg/mL. The sample preparation procedure in previous studies includes solid phase extraction (SPE)[40], liquid-liquid extraction (LLE), and protein precipitation. There is less interference on the chromatographs obtained by SPE or LLE method than by protein precipitation. However, sample preparation by SPE method consists of several steps. It is time-consuming and the SPE columns add the cost of determination. LLE method is also labor-intensive, and

large quantity of organic solvents used may be harmful. Although protein precipitation does not provide clean extractions like SPE and LLE, it is simpler, more rapid and more economical compared with SPE and LLE. Shipkova *et al*[42] used acetontrile, sodium tungstate and perchloric acid to precipitate protein. Khoschsorur *et al*[43] used 2 folds of acetontrile as the sample precipitation reagent. In the study by Chen *et al*[41], one fold of methanol containing 5% ZnSO₄ was used as the precipitation reagent. The procedure is very simple and rapid, and the result is reliable.

Determination of total MPA and its metabolites

As mentioned in the former part, MPA is metabolized primarily by glucuronidation to form MPAG and Ac-MPAG. Although MPAG is pharmacologically inactive, it can be hydrolyzed back to MPA and absorbed again during enterohepatic recirculation (EHC). AcMPAG has been observed regularly in the plasma of liver, kidney, and heart transplant recipients undergoing treatment with MMF. Chromatically based methods were established to monitor MPA, MPAG and AcMPAG simultaneously, including HPLC-UV methods[39-41] and LC-MS/MS methods[44,45]. To separate MPA from its metabolites sufficiently, both isocratic[41] and gradient[39,40] mobile phase systems were used. The peak areas of MPA, MPAG and AcMPAG at 304 nm were significantly lower than those at 215 nm (8.3, 21.8 and 9.4-fold lower, respectively) or 254 nm (2.0, 5.0 and 2.7-fold lower, respectively). Higher sensitivity was attained at 215 and 254 nm compared with 304 nm. However, the chromatography at 304 nm provided a cleaner baseline and more reproducible results in our study[41].

Klepacki *et al*[45] established an UHPLC-MS/MS assay using liquid-handling robotic extraction for the quantification of MPA and its metabolites in human plasma and urine. The LLOQ of MPA and its metabolites was 0.097 μg/mL for MPA and MPAG and 0.156 μg/mL for Ac-MPAG. The total assay run time was 2.3 min. The assay has proven to be robust and reliable during the measurement of samples from several pharmacokinetics trials.

Determination of total fMPA

The assays for detection fMPA are more complicated due to its very low level in plasma, therefore establishment of more sensitive methods is needed[46-49]. The pivotal sample treatment step is to separate fMPA from protein-bound MPA. Equilibrium dialysis and ultrafiltration can generate comparable results, and most studies selected ultrafiltration due to its practicability, accuracy and reproducibility. In the study by Aresta *et al*[46], plasma samples were ultrafiltrated in combination with SPE. The detection wavelength was UV 215 nm. The LLOQ was 26 ng/mL. Shen *et al*[47] used a HPLC-fluorescence method to determine total MPA and fMPA. The LLOQ of fMPA was 5 ng/mL. Chen *et al*[48] also developed a HPLC-fluorecence method to determine fMPA in plasma previously. The authors found that at a solvent pH of 8.5, the

LLOQ of fMPA reached 2.5 ng/mL, which was much lower than that of HPLC-UV and comparable with that of LC-MS/MS. The retention time of MPA was about 3 min when pH of the mobile phase was increased to 8.5. To prevent the endogenous interference, TBA was used as the ion-pair reagent[48].

The lower limit of assay sensitivity of LC-MS/MS made it the best choice in measuring fMPA concentration. Patel *et al*[49] established an LC-MS/MS assay, and the plasma was subjected to ultrafiltration followed by SPE using C18 cartridges. The assay has a LLOQ of 1 ng/mL and an accuracy > 95%. The method reported has an adequate degree of robustness and dynamic concentration range for the measurement of fMPA for TDM purposes or pharmacokinetics investigations. TDM of MPA in saliva offers a favorable non-invasive approach. Besides, concentration of MPA in saliva can be considered as the fMPA approximately. The LC-MS/MS assays for monitoring MPA in saliva were established for adult and pediatric patients.

Immunoassays

Immunoassays include a series of methods, and the mechanism of these methods is the competent combination of antibody between the MPA in plasma and labeled MPA. The most frequently used assay was commercial enzyme multiplied immunoassay technology (EMIT) assay. The advantage of being less labor intensive of EMIT rendered this assay more suitable for conventional clinical TDM. Although several studies revealed a 9%-15% of systematic positive bias between EMIT and HPLC assay, EMIT has been proven to be an efficient method for monitoring of MPA[50-52]. In the study by Chen *et al*[48] on liver transplant patients, 470 total MPA concentrations were determined by both HPLC and EMIT methods. The authors found the relationship of the two methods was EMIT = 1.074 × HPLC + 0.582 (r^2 = 0.918, *n* = 470, *P* < 0.05) for total MPA, and a good correlation between HPLC and EMIT was obtained with a positive bias of EMIT for total MPA (27.0%). The bias of EMIT is suggested to be caused by the cross-reactivity of Ac-MPAG.

Chen *et al*[48] established an EMIT method for the determination of fMPA for the first time. The calibration range of fMPA was 0.0050-0.50 μg/mL for EMIT method. Mean recovery of the two methods was 97.1%. The intra-day and inter-day variation coefficients were 4.51%-15.8% and 5.83%-19.5% for EMIT, respectively. The authors determined 297 fMPA concentrations by both HPLC and EMIT methods, and found that the relationship of the two methods was EMIT = 1.068 × HPLC + 0.004 (r^2 = 0.945, *n* = 297, *P* < 0.05), and a good correlation between HPLC and EMIT was obtained with a positive bias of EMIT for total MPA (23.3%). Although the LLOQ of EMIT is higher than that of HPLC method, more than 95% of fMPA samples determined by EMIT have concentrations higher than LLOQ. EMIT can also be used in monitoring of fMPA.

Other immunoassays include the cloned enzyme donor immunoassay, enzyme inhibition assay[53], and particle enhanced turbidimetric inhibition immunoassay[54]. These methods are either under-development or not widely used.

CHARACERISTICS OF PHARMACOKINETICS OF MPA

At present, a fixed dose of 1 or 1.5 g twice daily of MMF is the standard protocol in liver transplantation with adjustments only in relation to side effects or to its efficacy[11]. However, there are wide variations in MPA pharmacokinetics reported with standard MMF dosing in liver transplant recipients. Shaw *et al*[8] in his review reported that the range of MPA AUC was 5-160 mg.h/L in 22 liver transplant recipients receiving 1.0 g MPA, twice daily. This kind of variation has been confirmed in some studies in adult (Table 1) or pediatric liver transplantation[55].

The investigations for MPA pharmacokinetics in liver transplantation are focused on the early period after operation. There are several characteristics of MPA pharmacokinetics in early phase (about within 6 mo). First, mean MPA AUC will increase in a time dependent manner, especially in two or three weeks after liver transplantation. Second, a large range of intra-patient and/or within-patient MPA pharmacokinetic variability is observed. Third, the relationship between MMF dosage and MPA pharmacokinetic parameters is variable. Fourth, MPA exposure is different when different immunosuppressive drugs (TAC or CsA) are used.

Reasons of variation of MPA exposure may include type of recipient and donor graft, the process of liver transplantation, dosage of MMF, EHC, bowel, liver, and renal dysfunction and drug interactions.

Type of recipient and donor graft

In a control study by Jain *et al*[56], the MPA AUC in living donor liver transplant (LDLT) patients were 4-fold higher than in deceased donor liver transplant (DDLT) patients per 1 g MMF intravenously. The mean plasma concentration of MPAG was 1.4-2.0 times higher in deceased donor liver transplant patients compared with live donor liver transplant patients. A reduced size living donor graft may have lower metabolizing capacity and reduced glucuronidation activity during regeneration. Importantly, the authors suggested the need to use a lower dosage (approximately 30%) of MMF in live donor liver transplant patients compared with deceased donor liver transplant patients. Jain *et al*[57] showed a low bioavailability of oral MMF (mean, 48.5%, within 1 wk). The protocol using intravenous MMF can restore full bioavailability and conserve renal function after liver transplantation[58].

In another control study by Shen *et al*[59], the comparison of the pharmacokinetics of MPA and its metabolites between LDLT patients and DDLT patients was performed after oral administration of MMF (1 g, *bid*). Although the AUC$_{0-12h}$ of MPA and MPAG is not sig-

Table 1 Pharmacokinetic data of mycophenolic acid in adult liver transplant recipients

Ref.	Year	Regimen	Time since LT	n	Method	AUC_{0-12h} (mg.h /L)	Mean tmax (h)	Mean C_{0h} (mg/L)	Mean Cmax (mg/L)
Jain et al[65]	2001	TAC + MMF	Days 6-30	8	HPLC	40.0 ± 30.9 (7.3-102.3)	1.8 ± 1.6		10.6 ± 7.5
Mardigyan et al[92]	2005	TAC + MMF	> 12 mo	14	EMIT	45 ± 22	0.5	2.1 ± 1.5	12.2 ± 75
Pisupati et al[60]	2005	TAC + MMF	< week 1	10	HPLC	50.8 ± 42.1	1.8 ± 1.2		9.1 ± 7.2
			Weeks 1-2			60.3 ± 38.5	1.8 ± 1.4		11.6 ± 6.7
			Weeks 3-6			118.0 ± 57.6	1.3 ± 0.7		36.7 ± 15.6
Brunet et al[11]	2006	TAC + MMF	Day 6	13	HPLC-UC	17.4 (13.2-39.7)	2	0.4	4.6
			Day 16	13		26.3 (13.1-45.8)	1.2	0.6	7.7
			Month 3	14		33.6 (15.1-54.6)	0.7	1.3	6.6
Chen et al[71]	2007	TAC + MMF	Day 7	38	HPLC	44.6 ± 16.50 (17.99-96.87)	1.42 ± 0.77		8.45 ± 4.77
			Day 14	34		50.54 ± 18.60 (22.78-98.73)	1.45 ± 0.81		11.29 ± 5.51
Chen et al[76]	2008	TAC + MMF	Days 7-14	48	EMIT	45.77 ± 18.69 (10.66-117.01)	1.94 ± 1.65	2.02 ± 1.57	11.76 ± 6.34
Kamar et al[93]	2009	TAC + MMF	Day 7	15	HPLC	36.8 ± 27			
			Day 14	15		32.6 ± 11			
			Day 30	15		36.7 ± 13			
Beckebaum et al[94]	2009	TAC + MMF	Day 60 (14-230 d)	18	LC-MS/MS	55.9 (22.9-144.8)	0.5	3	14.2
		CsA + MMF	Day 70 (11-87 d)	12		52.2 (31.8-102.1)	1	2.5	15.3
Benichou et al[61]	2010	TAC + MMF	Day 12 (4-20 d)	26	EMIT	26.8 (21.8-39.7)			
			Day 36 (24-90 d)	25		45.2 (26.0-57.0)			

TAC: Tacrolimus; MMF: Mycophenolate mofetil; CsA: Cyclosporine A; HPLC: High-performance liquid chromatography; EMIT: Enzyme multiplied immunoassay technology.

nificantly different between the two groups, MPA AUC_{6-12h} was significantly higher in the DDLT group than in the LDLT group ($P < 0.05$). Inversely, higher free MPA AUC_{0-12h} and significantly higher free MPA fraction ($P < 0.05$) were observed in DDLT patients when compared with the DDLT group. AcMPAG AUC_{0-12h} was also significantly higher in the DDLT group ($P < 0.05$). The activity of glucuronide-conjugating enzymes was decreased due to reduced liver mass during the hepatic regeneration process. These observations suggested that the ability of clearance of MPA has decreased in LDLT patients during the early period after operation. The authors suggested that DDLT patients had higher EHC contributing to total MPA exposure compared with LDLT patients. As free MPA is the pharmacologically active form, lower oral dose of MMF may be administered for LDLT patients.

Post-transplant duration

MPA exposure significantly increases with post-transplantation time. In the investigation by Brunet et al[11] of 15 liver transplant recipients on a standard 1 g twice-daily dose, mean MPA AUC was 17.4 mg.h/L on day 6, 26.3 mg.h/L on day 10 and 33.6 mg.h/L at month 3. Low MPA AUC in their data was perhaps caused by the external biliary drainage and abnormal values of serum albumin and bilirubin. In another study by Xia et al[38], dose-normalized AUC_{0-12h} of MPA, MPAG and Ac-MPAG increased significantly in the later stage (> 1 mo) when compared with the data from the early stage (within 2 wk after liver transplantation). Pisupati et al[60] observed that MPA AUC_{0-12h} had doubled with 3-6 wk compared with that at first week after transplantation (50.8 mg.h/L vs 118 mg.h/L). However, the MPA AUC tended to be

stable after 3 to 6 mo. Benichou et al[61] showed that there is no change of MPA AUC or free MPA AUC between at mean 36 d (24-90 d) and at mean 867 d (124-6586 d).

The lower MPA AUC_{0-12h} in the immediate postoperative period is due to a higher apparent oral clearance (CL/F), which may result from a reduced absorption (F) or an increased clearance (CL). Benichou et al[61] assumed that the increase in CL/F is related to an increase in MPA free fraction, leading to lower total MPA AUC_{0-12h} value during the immediate postoperative period. Free fraction of MPA related well with MPA CL/F and decreased significantly as serum albumin level returned to normal, which would be consistent with more rapid hepatic and renal extraction, and subsequent biliary and urinary excretion. Pisupati et al[60] showed that total MPA CL/F decreased from 32.9 ± 21.4 L/h during the first week to 9.0 ± 4.4 L/h during 3-6 wk. The same authors also showed that there was no change in the intrinsic CL of MPA among the patients and suggested that the lack of a significant change in the intrinsic clearance indicates that the inherent ability of the liver to metabolize and eliminate MPA did not change significantly over time.

The other causes of low MPA exposure during the early stage may be related to the reduction of EHC and low bioavailability.

Dosage of MMF

The relationship between MMF dosage and MPA exposure is variable, usually weak or absent. In adult liver transplant recipients, Hwang et al[62] showed that there was a crude interindividual correlation between MMF dosage and MPA concentration ($r^2 = 0.271$, $P < 0.001$). When assorted according to the post-transplant period, r^2 was

0.153 during the first three months, 0.228 for months 4-12, 0.508 for years 1-2, 0.293 for years 3-5, and 0.247 after 5 years. With minimal TAC, a similar degree of inter-individual variation was observed ($r^2 = 0.247$, $P < 0.001$). In pediatric liver recipients, Aw *et al*[63] showed that MPA AUC_{0-7h} correlated significantly with MMF dose ($r = 0.552$, $P = 0.010$) and MPA C_{0h} ($r = 0.844$, $P < 0.001$). When assorted according to the post-transplant period, r^2 was 0.056 during the first three months, 0.162 for months 4-12, 0.085 for years 1-2, 0.071 for years 3-5, and 0.213 after 5 years.

EHC

MPA undergoes extensive EHC after hydrolysis of its biliary MPAG conjugate by intestinal bacteria and re-absorption of MPA. Hesselink *et al*[64] estimated that the contribution of EHC to the MPA AUC ranges between 10 % and 61 % in human. However, secondary peak is very rare in the initial period after liver transplantation, which occurs in approximately 50 % of patients at 1 mo[65]. In some liver transplant patients, the EHC reestablishes around 4 to 8 h after MMF dosage[66]. Pisupati *et al*[60] showed that a secondary peak in MPA was seen between 4 and 6 h after MMF administration in 4 of 10 patients during 3-6 wk and not seen during 1-2 wk. MPA AUC increased approximately 3-fold, which indicated the possible contribution of EHC. In pediatric liver recipients treated with CsA and MMF, Lobritto *et al*[55] observed that a second smaller peak was exhibited by some patients (probably due to EHC) although CsA was used, which decreased re-circulated MPA concentrations[67].

Impact of liver and renal dysfunction

Impairment of liver function has complex effects on MPA kinetics, although cirrhosis affects neither MPA absorption nor MPA plasma protein binding or pharmacokinetics[68]. It is believed that free MPA levels are affected by hypoalbuminemia, uremia and hyperbilirubinemia[8,69]. Free MPA levels increase markedly in patients with severe renal insufficiency[70].

Chen *et al*[71] showed that MPA AUC_{0-12h} in patients with abnormal albumin levels were significantly lower than that in patients with normal albumin levels ($P = 0.009$). MPA AUC_{0-12h} was related significantly with serum albumin levels ($r^2 = 0.412$, $P = 0.001$). However, other parameters of hepatic function including total serum bilirubin concentration did not influence the change of MPA AUC_{0-12h}. In 8 liver graft recipients, Jain *et al*[65] reported that MPA AUC correlated with serum bilirubin and MPA C_{0h} with albumin concentration. Higher serum bilirubin levels may impair hepatic MPAG production, transport and biliary excretion during cholestasis[68]. The decreased hepatic glucuronidation and EHC with moderate hepatic impairment may result in increased urinary MPAG concentrations[65]. Tredger *et al*[32] showed that recipients with low serum albumin levels (< 35 g/L) frequently failed to achieve the therapeutic levels of MPA. In adults and children with lower serum albumin concentrations, median levels of MPA C_{0h} were 42 % and 19 %, respectively, of

those in patients with normal serum albumin levels given corresponding doses ($P < 0.001$). However, Brunet *et al*[11] showed no relationship between liver function and MPA exposure.

Tredger *et al*[32] also reported that elevated serum creatinine levels (> 120 mmol/L) were related to higher MPA C_{0h} per unit MMF dose (median increase by 38% early and 50% late after transplantation, $P < 0.04$) only in adult patients.

Concomitant immunosuppressive drugs

CsA but not TAC decreased MPA AUC and increased MPAG AUC_{0-24h} because CsA inhibits excretion of MPAG into bile[67]. Inhibition of the biliary excretion of MPAG by CsA is mediated by the multidrug resistance-associated protein 2 transporter which leads to the reduction of MPA AUC[72].

In 21 stable pediatric liver transplant recipients, Brown *et al*[73] observed that MPA C_{0h} was significantly lower during co-therapy with CsA compared with co-therapy with TAC (2.8 mg/L *vs* 5.6 mg/L, $P = 0.006$), while MPAG AUC was correspondingly higher (229 mg/L/h *vs* 94 mg/L/h, $P = 0.012$). Higher MMF dosage was demanded with CsA to achieve equivalent MPA C_{0h} level than with TAC (362 mg *vs* 178 mg, $P = 0.004$). The authors suggested contrasting effects of CsA and TAC on MPA glucuronidation or its excretion and EHC.

Molina Perez *et al*[74] reported no interaction between total dose or BMI-adjusted dose of VGC and concomitant administration of MMF in liver transplant recipients.

LSS FOR MPA

Till now, there have been some studies establishing model equations for estimation of MPA AUC using LSS in liver transplant recipients.

Multiple regression analysis

The most reliable method for judging the exposure of MPA is to calculate MPA AUC_{0-12h}. But monitoring MPA AUC_{0-12h} requires frequent blood withdrawal. It is impractical to obtain 6-10 plasma samples for measuring full MPA AUC within a 12-h dose interval in clinical settings. Therefore, abbreviated sampling strategies by limited MPA concentrations have been under investigation.

For LSS study, Ting *et al*[75] have some important suggestions: (1) it is essential to validate the predictive performance of the LSS in other patient populations. The prediction bias and prediction precision of the LSS should be determined; (2) a clinically feasible LSS should use 3 or less blood samples, preferably within a short period of time in order to reduce the inconvenience of TDM; and (3) the application of a specific LSS is ideally limited to the population and drug formulation that is used to develop it.

Some studies tried to test whether MPA AUC can be accurately estimated from plasma concentrations at single time points, especially at MPA C_{0h}. However, it is very

Table 2 Limited sampling strategy for prediction of full mycophenolic acid area under the concentration-time curve in liver transplant recipients

Ref.	Method	Regimen	Patient population	No. of files (cases)	Sampling times investigated (h)	Suggested times of LSS (h)	Predicted AUC =	r^2	LSS validation	Bias	Precision
Attard *et al*[78]	EMIT or HPLC-UV	CsA or TAC + MMF	Pediatrics	41 files (41 cases)	0, 0.33, 0.67, 1.25, 2, 4, 6, 8	0, 0.33, 2	$9.1 + 5.7*C_{0h} + 1.1*C_{0.33h} + 2.1*C_{2h}$	0.740	No	N/A	N/A
						0.33, 0.67, 6	$5.2 + 7.1*C_{0h} + 1.1*C_{0.67h} + 5.4*C_{6h}$	0.880	No	N/A	N/A
Chen *et al*[71]	HPLC	TAC + MMF	Adults	72 files (40 cases)	0.5, 1, 1.5, 2, 4, 6, 8, 10, 12	1, 2, 4	$10.776 + 0.749*C_{1h} + 1.604*C_{2h} + 4.116*C_{4h}$	0.750	Validation	Yes	Yes
						1, 2, 6	$10.229 + 0.925*C_{1h} + 1.750*C_{2h} + 4.586*C_{6h}$	0.855	Group	Yes	Yes
						1, 2, 6, 8	$5.503 + 0.919*C_{1h} + 1.871*C_{2h} + 3.176*C_{6h} + 3.664*C_{8h}$	0.921		Yes	Yes
						1, 2, 4, 6	$6.658 + 0.921*C_{1h} + 1.573*C_{2h} + 2.057*C_{4h} + 3.543*C_{6h}$	0.899		Yes	Yes
Chen *et al*[76]	EMIT	TAC + MMF	Adults	48 files (48 cases)	0.5, 1, 1.5, 2, 4, 6, 8, 10, 12	1.5, 6	$10.56 + 1.55C_{1.5h} + 6.44C_{6h}$	0.859	Bootstrap	Yes	Yes
						2, 4, 8	$9.37 + 2.18C_{2h} + 2.10C_{4h} + 4.71C_{8h}$	0.901		Yes	Yes
						1, 2, 4, 8	$4.46 + 0.81C_{1h} + 1.78C_{2h} + 2.51C_{4h} + 4.94C_{8h}$	0.950		Yes	Yes
						1, 2, 4, 6	$5.92 + 1.10C_{1h} + 1.01C_{2h} + 1.77C_{4h} + 4.80C_{6h}$	0.927		Yes	Yes

LSS: Limited sampling strategy; MPA: Mycophenolic acid; AUC: Area under the concentration-time curve; EMIT: Enzyme multiplied immunoassay technology; HPLC: High-performance liquid chromatography; TAC: Tacrolimus; MMF: Mycophenolate mofetil; CsA: Cyclosporine A.

regretful that the relationship between MPA C_{0h} and MPA AUC_{0-12h} is not strong enough. In two studies by Chen *et al*[71] the r^2 value of MPA AUC_{0-12h} was also lower in monitoring MPA concentrations by HPLC ($r^2 = 0.300$, number of sample = 72) or EMIT ($r^2 = 0.0677$, number of samples = 48)[76] at the early stage after liver transplantation. In the study by Brunet *et al*[11], an acceptable correlation between MPA C_{0h} and MPA AUC_{0-12h} was found ($r = 0.742$, number of samples = 63). In pediatric liver transplantation, Brown *et al*[73] showed a moderate correlation between MPA C_{0h} and MPA AUC_{0-12h} ($r^2 = 0.65$, number of samples = 21). In conclusion, MPA AUC_{0-12h} could not be substituted correctly by MPA C_{0h} as well as other single time-point MPA concentrations.

Stepwise regression analysis was used to establish the abbreviated equations for estimated MPA AUC_{0-12h}. All combined models were obtained by using MPA concentrations at 1 to 4 time points. A number of regression equations that predict MPA AUC_{0-12h} are undertaken and take the form of the following function:

Estimated MPA $AUC_{0-12h} = I + \beta_1 \times C_{1h} + \cdots \beta_n \times C_{nh}$

Where I is intercept, β is partial correlation coefficient and C is MPA concentration. The largest r^2 value was considered the best regression. Equations with a high coefficient of determination (r^2) are then validated using data from another group or bootstrap procedure to evaluate their ability to predict the full MPA AUC. The validation step is critically important to assess reliability of the LSS. There are three main methods to validate an LSS: two-group (model-building group and validating group), bootstrap and jackknife methods.

Chen *et al*[71] developed an LSS for the prediction of MPA AUC using 72 profiles (40 cases) by HPLC (Table 2). These authors found that the relationship between estimated MPA AUC_{0-12h} and measured MPA AUC_{0-12h} based on three or four MPA pharmacokinetic parameters was related significantly in some abbreviated models. The best model for prediction of MPA AUC_{0-12h} was using MPA concentrations at 1, 2, 6 and 8 h time points ($r^2 = 0.921$, $P = 0.0001$). Bias and prediction are $1.24 \pm 11.19\%$ and $8.24 \pm 7.61\%$, respectively. 63 of 72 (88 %) estimated MPA AUC_{0-12h} values were within 15 % of MPA AUC_{0-12h}. Bland-Altman analysis also revealed the best agreement of this equation compared with the others and a mean error of ± 9.89 mg·h/mL. For validation of the accuracy of these equations, Hao *et al*[77] used another group of liver transplant recipients ($r^2 = 0.936$) with the excellent bias (2.18%). It was confirmed that the equation based on C_{1h}, C_{2h}, C_{6h} and C_{8h} had the best ability to predict measured MPA AUC_{0-12h} ($r^2 = 0.936$) with the excellent bias (2.18%), precision (5.11%) and the best prediction variation (2SD = ± 7.88 mg·h/L). However, the equation based on C_{1h}, C_{2h}, C_{6h} and C_{4h} was more suitable when considering clinical convenience, which had shorter sampling interval, excellent coefficient of determination ($r^2 = 0.795$), excellent bias (3.48%), acceptable precision (14.37%) and good prediction variation (2SD = ± 13.23 mg·h/L).

Although the standard technique for monitoring MPA concentration is HPLC, the EMIT has the advantages of convenience and rapidness in clinical settings for TDM of MMF. Due to the cross-reactivity of the antibody in the EMIT assay with the MPA AcMPAG, the EMIT target concentrations are higher than those for HPLC. The average overestimation by EMIT of MPA levels is approximately 10%-30%. As AcMPAG is pharmacologically active *in vitro*, it has been speculated that EMIT measurement may better

reflect immunosuppression than HPLC techniques that only measure the parent compound. Thus, establishment of the abbreviated model for estimation of full MPA AUC by EMIT method is necessary and valuable. Chen *et al*[76] established some equations for the prediction of MPA AUC using 48 profiles (40 cases) by EMIT (Table 2). The best equation was based on C_{1h}, C_{2h}, C_{4h} and C_{8h}. Forty of 48 (83.33 %) estimated MPA AUC_{0-12h} values were within 15 % of MPA AUC_{0-12h}. The bias and precision are 0.27% ± 1.79% and 8.83% ± 1.24%, respectively. The best agreement between estimated maximum a posteriori (MAP) AUC_{0-12h} and MPA AUC_{0-12h} was also showed by Bland-Altman analysis, with an average error of 9.02 mg.h/L. The authors conducted the Bootstrap analysis with 200 replicated datasets and confirmed the accuracy and robustness of this equation.

In two above investigations by Chen *et al*[71,76], MPA C_{6h} and/or C_{8h} were necessary in the best equations from MPA concentrations at 3 or 4 time points. The accurate equation by LSS should include one time-point MPA sample during the interval 6-12 h post-dosage. It is probable that in liver transplant recipients MPA EHC importantly contributed to the full MPA AUC.

In a study by Attard *et al*[78], a total of 41 MPA AUC_{0-8h} values were determined in 41 pediatric liver transplant recipients (Table 2). The best equation by LSS includes MPA C_{0h}, $C_{0.67h}$ and C_{6h} with excellent coefficient of determination ($r = 0.88$). For clinical practice, the equation with C_{0h}, $C_{0.33h}$ and C_{2h} is suitable ($r = 0.74$).

Bayesian analysis

MAP Bayesian assay is based on the concept that prior information or beliefs can be combined with observation data, which is known as Bayes' theorem[75,79]. Briefly, the priori population PK parameters, in combination with demographic, pathophysiological and limited concentration-time data from the individual, are used to predict the individualized parameters. Besides, the uncertainty of the parameters will also be estimated. As the amount of individual data accumulates, the population data contribute less to the overall prediction, and parameter prediction is individualized eventually. Prediction of parameters is achieved by minimizing the Bayesian Function:

$$Bayesian function = \sum \frac{(P_{pop} - \hat{P})^2}{var(P)} + \sum \frac{(C_{obs} - \hat{C})^2}{var(C)}$$

Where P_{pop} is the population average of parameter P; $\hat{P}$ is the individual expected average of parameter *P*; var(P) is the variance of the estimated parameter *P*; C_{obs} is the observed concentration value; $\hat{C}$ is the predicted concentration value; and var(C) is the variance of the predicted concentration[80].

Population pharmacokinetic study of MPA

A reliable Bayesian forecasting method is based on the reliability of population pharmacokinetic (PPK) models established. PPK parameters for commonly used drugs are available in popular Bayesian software programs (*e.g.*, NONMEM, ADAPT Ⅱ, PKS). PPK studies to date have mostly been undertaken in renal transplant recipients, with limited investigation in patients treated with MPA for autoimmune disease or haematopoietic stem cell transplantation. Most of these studies have involved use of the MMF formulation of MPA.

It is a hard work to develop a PPK model of MPA to fully describe the complex physiological processes that occur in relation to the absorption and EHC of this drug. There are more than 20 PPK models that have been developed for MPA, and more complex models for description of MPA pharmacokinetics also include modeling of metabolites and free MPA concentrations. However, most of these studies included less than 100 subjects, which are not sufficient to fully characterize the complex kinetics of this agent in different clinical conditions. Population models applied to MAP Bayesian analysis vary somewhat in structure, and separate covariates have been identified as being significant in different studies.

Sampling time of MPA PPK study varied between various studies, however, most studies using rich-time between two doses of MMF. The data also included various post-transplantation stages, and the longest time of sampling included data at 10 years post-transplantation[81]. The most frequently used structure model is 2-compartmental model. van Hest *et al*[82] collected data 3-140 d post-transplantation from 140 patients. A total of 6523 samples were obtained, and they tested 1-, 2- and 3-compartment models, and found that the 2-compartment model is most rational and suitable. Similar to other immunosuppressive agents, the absorption of MPA is very complex. Shum *et al*[83] tested different absorption models including first order absorption, time-dependent model, E_{max} model, Weibull model and dual sequential first order absorption process. Finally, first order absorption with a lag time improved the model significantly. Le Guellec *et al*[84] found a 2-compartment model with zero-order absorption, with the absorption duration being estimated from the data, provided the best fitting.

MAP Bayesian estimation of MPA AUC

After the final PPK model of MPA is obtained, the covariate values and selected concentration-time data from individual patients are input in the model to obtain individualized AUC. Most of studies used the trapezoidal method to estimate the full MPA AUC value, which is considered as reference value. Evaluations have been conducted of how closely MAP Bayesian estimation of MPA AUC matches.

External and internal validation methods can be used in the MAP Bayesian estimation of MPA AUC. External validation involves the application of the developed method to a new dataset, which requires the correct covariates and accurate sampling times recorded. It is more stringent in the study design and can provide the strongest evidence for evaluation. Most of studies evaluated using internal validation datasets through data splitting or using a re-sampling technique. In some studies, data were split into a population model-building group and a validation group to evaluate MAP Bayesian forecasting.

Other methods of validation include jackknife or Bootstrap method. Optimal sampling theory is based on the notion that there are specific sampling times, or windows of time, containing more information about pharmacokinetic parameters or drug exposure than other sampling times[85]. All these studies tested all combinations of study sampling times in selecting sampling times for Bayesian forecasting. Few studies used D-optimality (within predetermined time limits). Predictive performance is usually expressed in terms of the r^2, mean percentage predicted error (MPPE) and relative root mean-squared error (rRMSE) between reference AUC and estimated AUC.

A study by Barau *et al*[86] is the only study on the Bayesian estimation of MPA AUC in 28 pediatric patients who received liver transplantation. All patients received MMF therapy combined with TAC or CsA. The PPK model was established by using intensive pharmacokinetic datasets obtained from 16 children. A one-compartment model with first order absorption and first order elimination was selected. CL/F was estimated at 12.7 l h-1. Ka was estimated at 1.7 h^{-1} at age 8.7 years with IIV of 308%. V/F was 64.7 L, and increased about 2.3 times in children during the immediate post transplantation period. The individual MPA AUC$_{0-12h}$ was estimated by MAP Bayesian method using pharmacokinetic parameters obtained with the final model, including covariates, through Adapt Ⅱ software. The MPA AUC$_{0-12h}$ estimated from concentrations measured 0, 1 and 4 h after administration of MMF was in good correlation with the data obtained using the trapezoidal method.

MAP Bayesian estimation is more flexible compared with multiple linear regression methods. Drug exposure can be estimated with any number of blood samples taken at any time. Furthermore, with MAP Bayesian forecasting, the information about an individual patient may be helpful in the AUC estimation[87]. However, there are still some problems. First, the PPK model established for MAP Bayesian estimation may be not the best one for the limited cases. Second, the algorithms used to select the optimal sampling time may not be accurate enough. Third, there is still large bias in the prediction in various studies. Finally, the best sampling times by comparison of predictive performance cannot be regarded as truly optimal, because the possible combinations are limited by the study design. These problems should be solved by further studies before the method can be widely used in the individualized therapy with MPA.

CONCENTRATION-EFFECT RELATIONSHIP

It has been clearly shown that MMF is a very powerful immunosuppressive drug in preventing graft rejection. However, there was also plenty of evidence showing that MMF has serious side effects including hematologic and gastrointestinal disorders[4]. The prospective, randomized and double-blind trial performed by van Gelder *et al*[88] showed that the rate of acute rejection decreased significantly in renal transplantation if MPA AUC was in the target range of 32.2-60.6 mg.h/L. Although the results are conflicting among different transplant settings, MPA concentration monitoring is recommended in kidney transplantation by the therapeutic window of 30 to 60 mg.h/L for MPA AUC and of 1 to 3.5 mg/L for MPA C$_{0h}$[8]. However, it is still not widely accepted to individualize an oral MPA regimen by routinely monitoring MPA pharmacokinetic parameters in liver transplantation currently.

MPA exposure and acute rejection
In 147 adult liver transplants, Tredger *et al*[32] observed that nine of the 10 episodes of acute rejection were associated with plasma MPA concentrations less than 1 mg/L, with the exception occurring at 1.8 mg/L in a patient whose serum albumin was 31 g/L and creatinine 236 mmol/L. The relative risk of rejection (95%CI) increased 4.2-, 2.5-, and 1.6-fold, respectively, at plasma MPA concentrations of less than 0.5, 1.0 and 1.5 mg/L ($P = 0.003$, 0.002 and 0.058, respectively). The authors defined a cutoff of 0.85 mg/L in adult liver recipients by receiver operating characteristic (ROC) curve analysis. Besides, they also observed that MMF doses in the patients with rejection were not different from those in the control cohort. In the study by Hao *et al*[31], only two cases of acute rejection were proven by hepatic biopsy in 63 patients (3.2 %) within 3 mo after transplantation. Their MPA C$_{0h}$ values were 0.32 and 0.6 mg/L, MPA AUC$_{0-12h}$ values were 15.18 and 32.49 mg.h/L, and TAC C$_0$ values were 7.3 and 2.2 ng/L. Recently, Sarvary *et al*[89] found the optimal cutoff of MPA C$_{0h}$ for predicting acute rejection ($\geqslant$ 1.34 mg/L on CsA and $\geqslant$ 1.98 mg/L on TAC) in 56 liver transplant recipients during the 6-mo follow-up. In other studies, no relationship between MPA pharmacokinetics and acute rejection was established.

MPA exposure and adverse effects
In 63 liver transplant recipients, Chen *et al*[31] showed that mean MPA C$_{0h}$ and AUC$_{0-12h}$ in patients with side effects increased significantly compared with those without side effects (C$_{0h}$: 2.28 mg/L *vs* 1.31 mg/L, $P < 0.05$; AUC$_{0-12h}$: 49.68 mg.h/L *vs* 37.16 mg.h/L, $P < 0.01$). In addition, the levels of MPA C$_{0h}$ and MPA C$_{max}$ were higher in recipients with leucopenia, diarrhea and infection than in those without these effects, but a significant difference was achieved only during the episode of leucopenia (2.23 *vs* 1.81, $P < 0.01$). In 147 adult transplant recipients, Tredger *et al*[32] also showed that episodes of leukopenia were associated with higher median plasma MPA levels (2.8 mg/L *vs* 1.4 mg/L, $P = 0.004$). These authors also observed that MPA levels were higher during episodes of bacterial, fungal and viral infections, although this trend failed to achieve significance (1.8 mg/L *vs* 1.4 mg/L, $P = 0.056$) and there were no differences in median MPA levels with regard to gastrointestinal side effects. Brunet *et al*[11] showed significantly elevated mean MPA concentrations at C$_{0.66h}$ for six of 13 patients with diarrhea compared with symptom free patients (22.9 mg/L *vs* 7.4

Table 3 Receiver operating characteristic analyses of mycophenolic acid exposure and mycophenolate mofetil-related side effects in liver transplant recipients					
Ref.		Area under ROC curve	95%CI	Cut-off value	*P* value
Hao *et al*[31]	Side effects[1]				
	MPA C_{0h}	0.748	0.619-0.877	2 mg/L	0.001
	MPA AUC_{0-12h}	0.695	0.559-0.831	40 mg.h/L	0.012
	Leukopenia				
	MPA C_{0h}	0.670	0.534-0.805	2 mg/L	0.026
Tredger *et al*[32]	Leukopenia				
	MPA C_{0h}	0.780	0.642-0.919	2.25 mg/L	0.003
	MMF dose	0.750	0.662-0.837		0.007
	Infection				
	MPA C_{0h}	0.634	0.499-0.770	2.85 mg/L	0.056

[1]Side effects include leukopenia, diarrhea and infection. MMF: Mycophenolate mofetil; ROC: Receiver operating characteristic; MPA: Mycophenolic acid.

mg/L, $P < 0.05$) and there was no significant difference significantly in MPA C_{0h} or MPA AUC.

ROC curve analysis is also used to test the ability of MPA pharmacokinetic parameters to discriminate between cases with or without side effects in liver transplantation (Table 3). Hao *et al*[31] showed that the thresholds of MPA C_{0h} and MPA AUC_{0-12h} for side effects were 2 mg/L (sensitivity, 52.4%; specificity, 90.5%, $P = 0.001$) and 40 mg.h/L (sensitivity, 71.4%; specificity, 61.9%, $P = 0.012$), respectively. For individual side effects, only leukopenia was discriminated effectively by ROC analysis using MPA C_{0h} with a threshold of 2 mg/L (sensitivity, 56.5 %; specificity, 75 %, $P = 0.026$). The relative risks were 1.79 for MPA C_{0h} and 1.65 for MPA AUC to predict the occurrence of MMF-related side effects while 2.11 for MPA C_{0h} and 1.68 for MPA AUC to predict the occurrence of leukopenia. In the study by Tredger *et al*[32], corresponding more than 3-fold increases in the relative risks for leukopenia, infection and gastrointestinal disturbances were showed when MPA concentration was at 3 to 4 mg/L. The thresholds of MPA C_{0h} were 2.85 mg/L in infectious episodes (ROC area = 0.634, $P = 0.056$) and 2.25 mg/L in leukopenia (ROC area = 0.780, $P = 0.003$). Although the relative risk of gastrointestinal disorders increased with the increase in MPA C_{0h}, there was no significant association ($P > 0.5$). Importantly, the authors observed a significant association between MMF dose and episodes of leukopenia (ROC area = 0.750, $P = 0.007$). It is suggested that individualizing MMF dose instead of using a fixed dose might be helpful to optimize immunosuppression and minimize potential toxic effects. However, Hao *et al*[31] showed no significant difference in MPA pharmacokinetic parameters between patients with infection and those without.

Among immunosuppressive drugs, MMF is the main cause of diarrhea when compared with other agents. The mechanism responsible for MMF-related diarrhea is not yet elucidated. In liver transplantation[31,32], the levels of MPA C_{0h} or AUC_{0-12h} were not significantly higher in patients with diarrhea than those without diarrhea. How-

ever, Xia *et al*[38] found that MPA C_{6h}, C_{10h}, C_{12h} and MPA AUC_{6-12h} were significantly higher in patients with diarrhea ($P < 0.05$). These results suggested that higher EHC might contribute to the occurrence of diarrhea.

It was guessed that diarrhea may be related to MPAG or AcMPAG[90]. However, in the study by Xia *et al*[38], there was no significant difference in MPAG or AcMPAG ($P > 0.05$) though MPA C_{max} and MPA AUC_{0-12h} of MPAG were higher in recipients with diarrhea. Likewise, C_{0h}, C_{max}, and AUC_{0-12h} of AcMPAG were also higher in patients with diarrhea, although no significant difference in these parameters was found ($P > 0.05$). Arns *et al*[91] suggested that the capacity of enterocytes to participate in MPA metabolism could potentially result in local generation of AcMPAG and MPAG with consequent direct toxic effects on the gastrointestinal tract. Perhaps concentration of AcMPAG in the gastrointestinal tract is more important than plasma concentration of AcMPAG for induction of diarrhea.

Another risk of diarrhea was dependent on dosage of MMF. Diarrhea was controlled by decreasing the dosage or interruption even if these patients had the same starting dosage of MMF as those not suffering from diarrhea[31].

CONCLUSION

Until now, TDM for MPA has not been fully accepted in liver transplantation as no long-term prospective study of concentration controlled *vs* fixed-dose prescribing of MMF has been done. However, based on published data, it is confirmed that intra- or inter-individual MPA pharmacokinetic variability exists, which is related to greater risk of acute rejection at lower MPA concentrations and MMF-associated side effects at higher MPA concentrations. On the other hand, the standard dose of MMF is rarely necessary in liver transplant recipients who had more MMF-related side effects and less acute rejection. These data suggest that monitoring MPA exposure is helpful in clinical settings.

In liver transplantation, it was showed that MPA C_{0h} has more practical benefits over MPA AUC although the relationship between MPA C_{0h} and MPA AUC is not very strong in some studies. Compared with the therapeutic window in renal transplantation (MPA C_{0h}: 1-3.5 mg/L), acute rejection is more likely at concentrations less than 1 to 2 mg/L (μg/mL) and adverse effects at concentrations 3-4 mg/L or greater in liver transplantation[13]. However, this finding needs more clinical validation in future. Although MPA AUC is much accurate, which reflects the change of MPA pharmacokinetics and is closely related to side effects[31], no recommended therapeutic ranges of MPA AUC could be used in pediatric or adult liver transplant recipients. On the other hand, monitoring of MPA AUC is not practical in clinical settings. It should obtain 6-10 plasma samples for measuring full MPA AUC within a 12-h dose interval. Although abbreviated sampling strategy by limited MPA concentrations is practical in clinical settings, the equations including MPA concentra-

tions within 2 h with good correlation were only seen in pediatric transplant recipients[78]. In adult liver transplantation, good coefficients of determination (r^2) were seen in equations including one MPA concentration at least during 6-12 h after oral MMF[71,76]. Monitoring MPA C_{0h} has more practical benefits than MPA AUC in liver transplantation.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Involvement of eicosanoids in the pathogenesis of pancreatic cancer: The roles of cyclooxygenase-2 and 5-lipoxygenase

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Abstract

The interplay between inflammation and cancer progression is a growing area of research. A combination of clinical, epidemiological, and basic science investigations indicate that there is a relationship between inflammatory changes in the pancreas and neoplastic progression. Diets high in ω-6 polyunsaturated fatty acids provide increased substrate for arachidonic acid metabolism by cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) to form eicosanoids. These eicosanoids directly contribute to pancreatic cancer cell proliferation. Both COX-2 and 5-LOX are upregulated in multiple cancer types, including pancreatic cancer. *In vitro* studies using pancreatic cancer cell lines have demonstrated upregulation of COX-2 and 5-LOX at both the mRNA and protein levels. When COX-2 and 5-LOX are blocked *via* a variety of mechanisms, cancer cell proliferation is abrogated both *in vitro* and *in vivo*. The mechanism of COX-2 has been shown to include effects on apoptosis as well as angiogenesis. 5-LOX has been implicated in apoptosis. The use of COX-2 and 5-LOX inhibitors in clinical studies in patients with pancreatic cancer has been limited. Patient enrollment has been restricted to those with advanced disease which makes evaluation of these drugs as chemopreventive agents difficult. COX-2 and 5-LOX expression have been shown to be present during the early neoplastic changes of pancreatic cancer, well before progression to invasive disease. This indicates that the ideal role for these interventions is early in the disease process as preventive agents, perhaps in patients with chronic pancreatitis or hereditary pancreatitis.

Core tip: This review article highlights the relationship between inflammation and pancreatic cancer, specifically focusing on the enzymes cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX). The role of inflammation and tumor progression is a burgeoning area of research. This review delves into the research that has been conducted investigating COX-2 and 5-LOX and their relationship to pancreatic cancer both *in vivo* and *in vitro*. We discuss a variety of investigations including basic science, epidemiological, and clinical as they relate to pancreatic inflammation and eicosanoids.

Knab LM, Grippo PJ, Bentrem DJ. Involvement of eicosanoids in the pathogenesis of pancreatic cancer: The roles of cyclo-oxygenase-2 and 5-lipoxygenase. *World J Gastroenterol* 2014; 20(31): 10729-10739 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i31/10729.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i31.10729

INTRODUCTION

The relationship between inflammation and cancer is well established. Rudolf Virchow noticed leukocytes in cancerous tissue as early as 1863 and conjectured that there was a link between chronic inflammation and neoplasia[1]. This theory has been validated by clinical examples such as Marjolin's ulcers which are squamous cell carcinomas that form in sites of chronic inflammation such as burn scars or chronic ulcers[2]. Other examples of inflammatory conditions with correlative cancers are inflammatory bowel disease and colorectal cancer, gastritis caused by *Helicobacter pylori* and gastric cancer, hepatitis and hepatocellular carcinoma, and chronic pancreatitis and pancreatic cancer. These examples highlight the impact of inflammation on the neoplastic process though the mechanism is unclear.

The inflammatory response is marked by cytokine release from epithelial cells which attract and activate inflammatory cells. When macrophages, neutrophils, fibroblasts, and mast cells are attracted to this inflammatory microenvironment, they produce reactive oxygen species (ROS) and stimulate epithelial cell proliferation[3]. The infiltration of these cells into the tumor microenvironment has been implicated in pancreatic tumor progression (Figure 1)[4-7]. ROS can directly cause DNA damage by increasing the probability that genetic mutation will occur. Combined with their effects on cellular proliferation, ROS increase the likelihood of neoplastic transformation[3,8]. A key step in the inflammatory process is the activation of the arachidonic acid pathway that produces eicosanoids. The purpose of this paper will be to review inflammatory mechanisms as they relate to pancreatic cancer, specifically the roles of cyclooxygenase (COX) and lipoxygenase (LOX), and how their metabolites contribute to carcinogenesis.

INFLAMMATION AND PANCREATIC CANCER

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States, and the vast majority of those afflicted succumb to this disease. The 5-year survival rate is about 5%-6%[9]. Since the majority of pancreatic cancer is discovered late in the disease process, well after potentially curative surgery is an option, understanding the early oncogenic changes is necessary to aid in prevention. Since inflammation has been shown to be a key factor in the neoplastic process as it contributes to genetic changes and DNA damage, its role in pancreatic cancer is of particular interest.

Studying the mechanisms of pancreatitis in patients can be helpful for understanding inflammation as it relates to pancreatic cancer development. Patients with hereditary pancreatitis, a rare disease responsible for less than 1% of pancreatitis cases, have frequent episodes of acute inflammation[10]. Repeated episodes of pancreatitis result in fibrosis, chronic inflammation, and the eventual destruction of the gland[11]. This chronic inflammatory environment is thought to contribute to malignant transformation of pancreatic ductal cells. In patients with hereditary pancreatitis, the risk of developing pancreatic cancer is 53 times higher than unaffected individuals, and by 70 years of age, approximately 40% of these patients will develop pancreatic cancer[10]. Patients afflicted with non-hereditary chronic pancreatitis also have an increased risk of pancreatic cancer. Population studies suggest that patients with chronic pancreatitis are 17 times more likely to develop pancreatic cancer compared to age matched controls, and the risk is correlated with the duration of inflammation[12]. Therefore it will be important to understand the mechanisms that link pancreatitis to the development of pancreatic cancer.

The inflammatory process begins with the inappropriate release of proteolytic pancreatic enzymes that cause acinar cell injury[13]. This generates an immune response in which inflammatory cells are attracted to cytokines released from the cells at the site of injury. Our lab, as well as others, previously investigated the relationship between one of the major inflammatory cell types, mast cells, and pancreatic cancer[6,14]. We have shown that mast cell infiltration in pancreatic cancer specimens correlates with worse prognosis[6]. Ma demonstrated that pancreatic ductal adenocarcinoma (PDAC) cells promote mast cell migration and activation *in vitro*. The study also showed that blocking mast cell migration in an orthotopic PDAC mouse model decreased PDAC growth *in vivo*[15]. Similarly, Soucek demonstrated in an islet-cell tumor mouse model that mast cells mediate expansion of these tumors and are essential for tumor maintenance[5].

The generation of ROS and activation of the arachidonic acid pathway are also key steps in potentiating the inflammatory response[13]. The body mounts a natural response to chronic insults to the pancreas by releasing growth factors such as platelet-derived growth factor and transforming growth factor beta. This stimulates cell proliferation, which can potentially worsen DNA damage and increase genetic mutations[16].

EPIDEMIOLOGICAL STUDIES

Epidemiological studies have shown that high-fat diets, specifically with a high proportion of polyunsaturated omega-6 fatty acids, are associated with increased cancer rates, particularly in breast, pancreas, and prostate cancers[17-22]. Studies have shown that cancer incidence in an ethnic group often changes after migration and drastic dietary changes. An example is the migration of the Japanese to Western countries that have relatively higher fat diets compared to Japanese diets. Studies have reported increased colon, pancreas, breast, and prostate cancer incidence in individuals migrating to Western countries from Japan[21]. The relationship between a high-fat diet and pancreatic cancer was evaluated by a prospective study investigating obesity in various age groups including early adulthood, midlife, and older age. There were
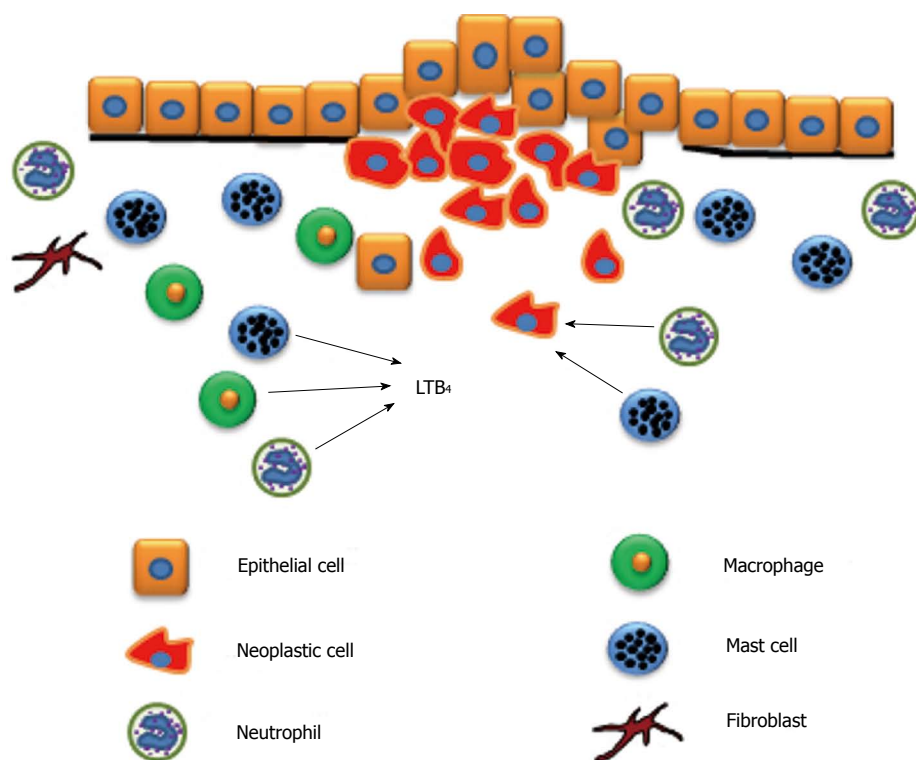
Figure 1 Inflammatory cell infiltration into the tumor microenvironment. As pancreatic adenocarcinoma progresses, inflammatory cells such as mast cells, neutrophils, and macrophages are attracted to the tumor microenvironment and enhance tumor growth. Leukotriene B4 (LTB4) is a chemotactic factor for macrophages, neutrophils, and mast cells. Fibroblasts are also activated and enhance collagen production.

significant positive associations between pancreatic cancer and obesity in all age groups studied[23]. Patients with the longest duration of obesity and diabetes were at the greatest risk for pancreatic cancer[23]. One of the mechanisms proposed for this association is the high content of arachidonic acid in animal fats. Arachidonic acid is metabolized to biologically active lipids by COX, LOX, and epoxygenase pathways to generate eicosanoids[24]. Eicosanoids have been implicated in various carcinogenic mechanisms including tumor progression and metastasis[25]. Studies conducted in EL-Kras transgenic mice fed a high ω-6 fatty acid diet demonstrated increased frequency and size of pancreatic neoplastic lesions as well as increased pancreatic mast cell densities[26]. In a related study, a high ω-3 fatty acid diet in EL-Kras transgenic mice was found to have a protective effect against the formation of pancreatic lesions. These mice had reduced incidence, frequency, and proliferative index of pancreatic precancer compared to those fed standard chow[27]. In unpublished findings by our lab, we demonstrate that EL-Kras transgenic mice fed high ω-6 fatty acid diets had increased PGE2 and LTB4 compared to their counterparts fed a high ω-3 fatty acid diet. Therefore, ω-3 and ω-6 fatty acids are involved in carcinogenic mechanisms and have opposing effects on pancreatic neoplasia, which is hypothesized to be mediated through the regulation of eicosanoid production.

Further evidence to support the role of eicosanoids in the carcinogenic process are epidemiological studies indicating that the use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the incidence of various solid tumors[24,28]. One study used a meta-analysis to examine the effect of regular NSAID use on colon, lung, breast, and prostate cancers. The results indicated that there is a risk reduction of 43% for colon cancer, 28% for lung cancer, 25% for breast cancer, and 27% for prostate cancer[28]. The role of NSAIDs and pancreatic cancer is not clear. Anderson conducted a prospective study with 28000 post-menopausal women and demonstrated a decreasing trend in pancreatic cancer incidence in women with more frequent aspirin use[29]. Alternatively, a study among United States adults followed for 18 years found no association between aspirin use and pancreatic cancer mortality[30]. A different prospective study in a large cohort of women with an 18 year follow-up showed an association with long-term aspirin use and pancreatic cancer although there was a higher prevalence of obesity and diabetes mellitus among patients who reported regular aspirin use[31]. A study conducted in the United Kingdom demonstrated that NSAID use for more than 773 d in the 5 years prior to diagnosis was associated with a 20% risk reduction of pancreatic cancer, although increasing doses did not have an impact on risk[32]. A meta-analysis involving 11 studies analyzing the association between pancreatic cancer and aspirin and other NSAIDS did not find a conclusive association[33]. The summary relative risk did not find an association between aspirin or other NSAIDS and pancreatic cancer, nor an association between regular use vs irregular use, nor frequency of aspirin or NSAID use[33].
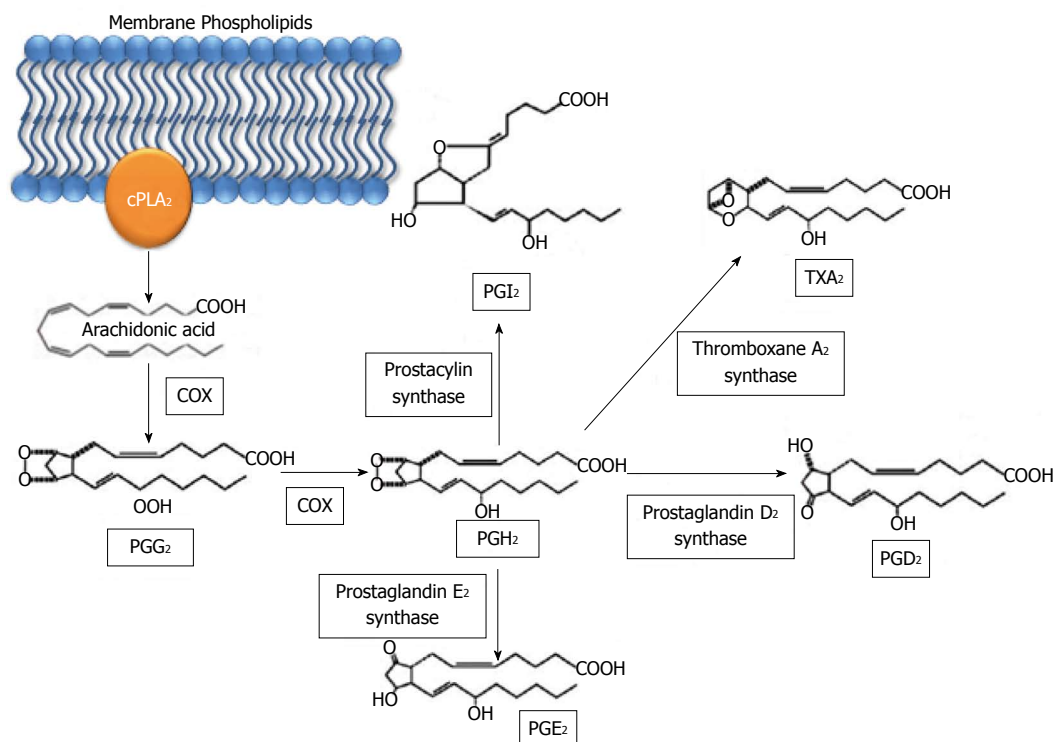
Figure 2 Metabolic pathway of prostaglandins *via* cyclooxygenase. Arachidonic acid is released from membrane phospholipids by phospholipase A_2 and converted to PGG_2 and subsequently PGH_2 by COX. PGH_2 is then converted to PGI_2, TXA_2, PGD_2, and PGE_2. cPLA2: Cytosolic phospholipase A_2; COX: Cyclooxygenase; PG: Prostaglandin; TX: Thromboxane.

BIOCHEMISTRY OF COX AND LOX

The ability of NSAIDs to exert their anti-inflammatory and anti-tumor effects by inhibiting the COX enzyme, which results in decreased prostanoid production, demonstrates the intimate relationship between inflammation and cancer[24]. There is evidence to suggest that 5-LOX, a close relative of COX-2, is essential for eicosanoid production and tumor pathogenesis. The precursors of eicosanoids are arachidonic acids. Both prostaglandins (PG) and leukotrienes (LT) are members of the eicosanoid family, which are lipid mediators made of a 20 carbon fatty acid derivative[34]. Eicosanoids are vital due to their distinct biological activity in the body and effectiveness in the nanomolar concentration range[34]. The two eicosanoid members that will be discussed in detail here are prostaglandins and leukotrienes.

Prostaglandins are made by most cells in the body, and they act as both paracrine and autocrine mediators[34]. Arachidonic acid is released from the membrane by the phospholipase cPLA2 and acted on by prostaglandin G/H synthase (known as COX) to become an intermediate known as PGH_2[24] (Figure 2). There are two main forms of COX: COX-1 and COX-2. COX-1 is generally thought of as the constitutively expressed enzyme that is responsible for basal production of prostanoids for tissue homeostasis, and COX-2 is induced by cytokines and growth factors, particularly at sites of inflammation and neoplasia[13]. Therefore, COX-2 has a key role in the setting of inflammation and the tumor microenvironment[24].

Leukotrienes, while derived from the same precursor as prostaglandins, are functionally distinct. Leukotrienes are predominately produced by inflammatory cells, and once cellular activation occurs, cPLA2 and 5-lipoxygenase (5-LOX) are translocated to the nuclear envelope[34]. LOX enzymes are a family of nonheme iron-containing dioxygenases with labeling based on the location of oxygen insertion at the carbon position of arachidonic acid[25]. The most common LOX enzymes are 5-, 8-, 12-, and 15-LOX[25]. These then form the corresponding hydroperoxyeicosatetraenoic acids (HPETE)[25]. Specifically, 5-LOX transforms arachidonic acid *via* a dehydration reaction to the unstable epoxide LTA_4[25]. LTA_4 is further oxidized to form either 5-HETE or the leukotrienes[25]. LTA_4 can be hydrolyzed by leukotriene A4 hydrolase in the cytoplasm or nucleus resulting in LTB_4 (Figure 3). LTB_4 is known as a potent chemoattractant, and its receptors are upregulated in pancreatic cancer[35]. LTA_4 can also be conjugated with glutathione to form LTC_4 by LTC_4 synthase. LTC_4 can then undergo extracellular metabolism resulting in LTD_4 and LTE_4[34]. The activation of 5-LOX is dependent upon the 5-LOX-activating protein (FLAP).

One of the ways in which LTB_4 directs chemotaxis and regulates neutrophil adhesion is by activating integrin receptors[34,36-39]. It has been demonstrated that local cell death causes "swarm-like" interstitial neutrophil clustering and LTB_4 plays an important role in intercellular communication between neutrophils and facilitates neu-
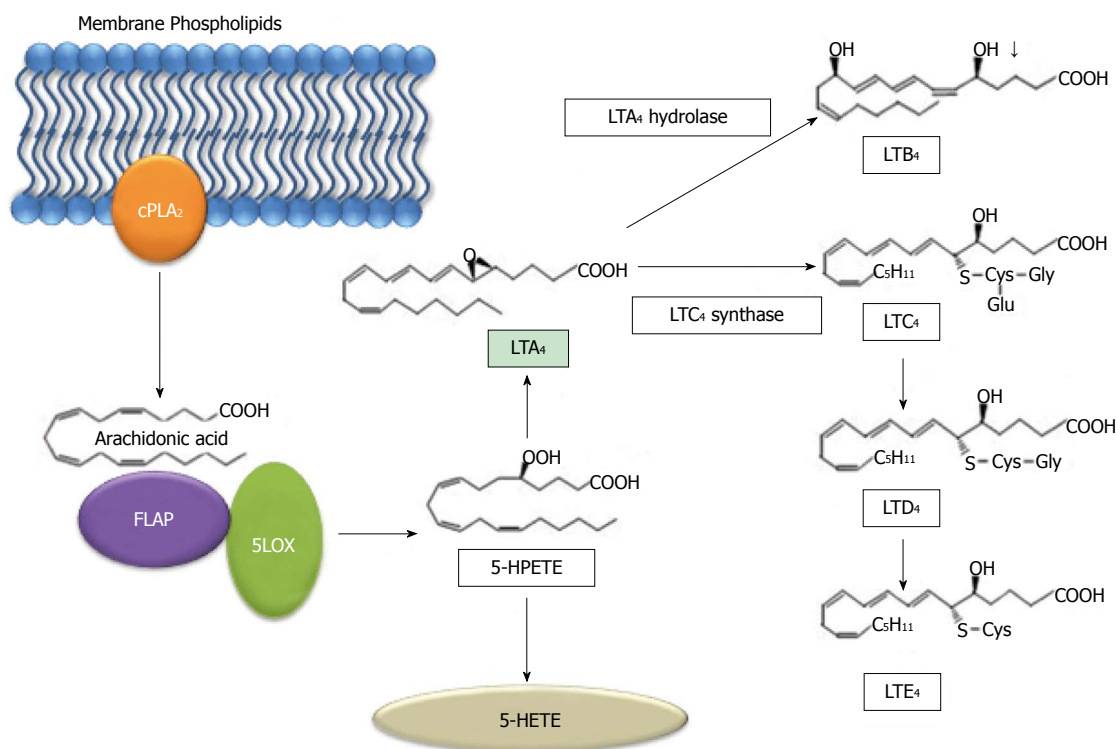
Figure 3 Metabolic pathway of arachidonic acid *via* 5- lipoxygenase. Arachidonic acid is released from membrane phospholipids by phospholipase A_2 and converted to 5-HPETE by 5-LOX and 5-LOX activating protein (FLAP). 5-HPETE can then form either 5-HETE or LTA_4. LTA_4 then becomes LTB_4 or LTC_4. LTC_4 can then form LTD_4 and subsequently LTE_4. cPLA2: Cytosolic phospholipase A_2; LOX: Lipoxygenase; FLAP: 5-LOX activating protein; HPETE: Hydroperoxyeicosatetraenoic acid; HETE: Hydroxyl 6 *trans* 8, 11, 14 *cis* eicosatetraenoic acid; LT: Leukotriene.

trophil movement through tissue[39]. In the tumor microenvironment, LTB_4 has been shown *in vivo* to enhance leukocyte recruitment into the tumor stroma[40].

ROLE OF COX IN PANCREATIC NEOPLASIA AND CANCER

COX-2 expression is upregulated in a variety of malignancies including colon, esophagus, breast, and pancreatic cancer[41-43]. Multiple studies have indicated that COX-2 is also important in carcinogenesis. One example in a murine model of familial adenomatous polyposis showed a marked reduction in the number and size of intestinal polyps in COX-2 null mice with an APC mutation[44].

The relationship between COX-2 and pancreatic cancer has been evaluated in multiple studies with the majority of the evidence demonstrating upregulated COX-2 expression in pancreatic cancer at both the mRNA and protein levels. One study showed that levels of COX-2 mRNA were increased 60-fold in pancreatic cancer compared to normal tissue. In addition, COX-2 protein was expressed in 9 out of 10 pancreatic cancer samples, while nontumor samples had no COX-2 expression[45]. Immunohistochemistry (IHC) confirmed COX-2 expression in malignant epithelial cells[45]. A different study demonstrated an increase in COX-2 expression using IHC when pancreatic carcinoma was compared to normal pancreas[43]. Five pancreatic cancer cell lines were studied, and COX-2 protein expression was detected in BxPC-3, Capan-1, and MDAPanc-3 cells, and increased levels of COX-2 mRNA were detected in 4 of the 5 cell lines[43]. When an NSAID was used, a dose-dependent inhibition of cellular proliferation was observed in all cell lines studied[43]. Kokawa *et al*[46] used different pancreatic cancer cell lines (KP-2, PNS-1, MiaPaca-2, and Panc-1) to show that COX-2 expression was upregulated in all 4 of them, and NSAID inhibition of cellular proliferation was correlated with the expression of COX-2. Maitra used automated cellular imaging to evaluate COX-2 expression not only in pancreatic adenocarcinoma but also its precursor, pancreatic intraepithelial neoplasia (PanIN). This showed an increase in the overall average number of positive cells from 19.2% in normal ducts to 36.3% in PanINs to 47.3% in adenocarcinomas[47]. This study suggests tumorigenic activity of COX-2 in preinvasive pancreatic lesions and a potential role for chemopreventive agents such as COX-2 inhibitors in pancreatic cancer.

While multiple studies have shown the association between pancreatic cancer and COX-2 expression, few have investigated the underlying mechanism of COX-2 and how it promotes neoplastic changes. Overexpression of COX-2 leads to increased tumor prostanoid levels, and PGE_2 is known to have several tumorigenic effects. PGE_2 has been implicated in the inhibition of apoptosis and the induction of proliferation and angiogenesis[48]. One group investigated the relationship between high-mobility group A1 (HMGA1) and COX-2 in pancreatic

cancer. The authors proposed that the HMGA1-COX-2 axis is a key molecular pathway in pancreatic cancer because the upregulation of COX-2 expression is HMGA1 dependent in various pancreatic cancer cell lines. It was first demonstrated that a positive correlation between HMGA1 and COX-2 expression in six pancreatic cancer cell lines (BxPC-3, HPAF-Ⅱ, MiaPaCa-2, Panc1, PL45, and XPA-3) existed[49]. COX-2 expression after knock-down of HMGA1 in two pancreatic cancer cell lines was evaluated and showed that HMGA1 binds to the COX-2 promoter to induce its expression[49]. A significant reduction in COX-2 expression after using an HMGA1 siRNA was observed, and COX-2 inhibitors blocked tumorigenesis in human pancreatic cancer xenografts that overexpressed HMGA1[49].

Another potential mechanism proposed for the involvement of COX-2 in tumorigenesis is its effect on angiogenesis. Chu compared the angiogenic effects of a COX-2 expressing pancreatic cancer cell line BxPC-3 with the COX-2 negative AsPC-1 cell line. The group found a significant increase in endothelial cell migration induced by BxPC-3 migration compared with AsPC-1. These findings were supported by data demonstrating that BxPC-3 treatment with a COX-2 inhibitor decreased the angiogenic responses of the endothelial cells[50]. Eibl *et al*[51] showed in a subset of pancreatic cancer cell lines that COX-2 increased PGE2 which subsequently increased VEGF secretion. In a subsequent *in vivo* study, an orthotopic pancreatic cancer model in nude mice was used to demonstrate the effects of nimesulide, a selective COX-2 inhibitor, on angiogenesis. In mice with COX-2 positive tumors, nimesulide resulted in an increase in VEGF production by malignant cells but a compensatory decrease in production by nonmalignant cells, ultimately leading to reduced tumor angiogenesis and growth[52].

Ito's study on the effect of COX-2 on tumor invasion found that PGE2 mediated pancreatic cancer cell invasion through induction of matrix metalloproteinase-2 expression. This induction was found to be dependent on an extracellular signal-regulated kinase (ERK)/Ets-1-dependent mechanism[53].

Another study investigated the expression of COX-2 on clinical outcomes and found no correlation between global COX-2 expression and clinical outcome. The clinical outcomes studied were survival, stage, tumor size, or vascular invasion[54]. The expression of COX-2 was related to an increase in perineural invasion[54].

Several preclinical mouse models evaluating pancreatic lesions have been reported. One particular transgenic model, LSL-KRASG12D; PDX-1-Cre, is a mouse with a KRAS mutation expressed in pancreatic progenitor cells. This model results in PanIN lesions which eventually develop through advanced PanIN lesions into adenocarcinoma[55]. The efficacy of a selective COX-2 inhibitor, nimesulide, was evaluated in this mouse model. Animals treated with nimesulide demonstrated significantly fewer PanIN lesions and decreased intrapancreatic prostaglandin E2 levels compared to mice on a control diet[55].

In two unpublished works from our group, another mouse model with mutant Kras expression targeted to acinar cells (EL-Kras)[56] have been crossed with COX-2 knock-out mice to generate cohorts of EL-Kras/COX-2-/- mice. These mice have a significantly reduced frequency of cystic papillary neoplasms compared with EL-Kras mice with wild-type COX-2. Also, mice that overexpress COX-2 in acinar cells develop hyperplastic, mildly dysplastic ducts with accompanying focal fibrosis and lymphocytic infiltration[57]. A different transgenic mouse model, BK5.COX2, results in COX-2 overexpression in the exocrine pancreas[58]. The resulting histology demonstrated pancreatitis-like changes with acinar-to-ductal metaplasia by 3 mo, and at 6-8 mo strongly dysplastic features. The described phenotype was completely prevented by maintaining the mice on a COX-2 inhibitor. Cell lines derived from lesions in these mice were tumorigenic when injected into nude mice. Both of these mouse models highlight the relationship between COX-2 and pancreatic cancer and will be important in future studies.

ROLE OF LOX IN PANCREATIC NEOPLASIA AND CANCER

Similar to COX-2, LOX has been implicated in several human cancers including lung, prostate, colon, breast, and pancreatic; however, relatively little research has been conducted to elucidate its role in cancer progression[59-61]. 5-LOX expression is upregulated in both pancreatic adenocarcinoma as well as in neoplastic lesions of the pancreas[25]. In a study by Hennig, three pancreatic cancer cell lines, AsPC-1, PANC-1, and MiaPaCa2, were found to have 5-LOX mRNA expression while normal human pancreatic cells did not express 5-LOX[35]. They also confirmed that 5-LOX protein was expressed in these cell lines and in two additional cell lines, Capan-1 and HPAF[35]. Moreover, the expression levels of both 5-LOX and its downstream metabolite LTB4 were found to be significantly upregulated in pancreatic tumors compared with normal pancreatic tissue[35]. Interestingly, staining was evident in both the cancer cells as well as the ductal cells and adjacent islets. A follow-up study by Hennig *et al*[62] investigated 5-LOX expression in PanIN lesions. Greater than 90% of the ductal cells had strong positive 5-LOX staining in all grades of PanINs with no significant difference between grades of PanINs. This was compared to normal pancreatic specimens that had 0 to 7.5% of the ductal cells showing 5-LOX staining[62]. This study also reported that 5-LOX expression was present in pancreatic PanIN-like lesions in N-nitroso-bis (2-oxopropyl)-amine (BOP) treated hamsters as well as EL-Kras transgenic mice[62]. Ding reported similar results showing increased 5-LOX expression in MiaPaCa2, PANC-1, AsPC-1, and Capan2 pancreatic cancer cell lines at the mRNA level[63]. The general LOX inhibitor (NDGA), a 5-LOX inhibitor (Rev5901), and a FLAP inhibitor (MK-886), all inhibited thymidine incorpora-

tion in MiaPaCa2 cells indicating that these compounds induced growth inhibition in pancreatic cancer cells. Finally, it was demonstrated that arachidonic acid and linoleic acid induced pancreatic cancer cell proliferation[63].

While there have been no studies published to date examining mouse models deficient in 5-LOX, our lab is currently investigating this mouse model. We have developed a EL-Kras/5-LOX null mouse and preliminary results have indicated a decrease in pancreatic lesions in the 5-LOX null mice compared with their wildtype counterparts.

While it is well established that 5-LOX plays an important role in pancreatic tumor progression, fewer studies have investigated its underlying mechanism in this disease. Ding showed that the 5-LOX metabolite, 5(S)-hydroxyeicosatetraenoic acid [5(S)-HETE], stimulates pancreatic cancer cell proliferation in a time- and concentration-dependent manner[63]. In a subsequent study, Ding demonstrated that 5-(S)-HETE has mitogenic effects due to its role in the MEK/ERK and PI3 kinase/AKT pathways[64]. In an additional study, this group demonstrated that both the general LOX inhibitor (NDGA) and the 5-LOX inhibitor (Rev5901) induced apoptosis in four different pancreatic cancer cell lines[65]. Apoptosis was confirmed using three different methods including DNA propidium iodide staining, DNA fragmentation, and terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay in PANC-1, MiaPaCa2, Capan2, and HPAF cell lines[65]. A follow-up study performed by Tong further delineated the mechanism behind the LOX inhibitor-induced apoptosis showing that it is a mitochondria-mediated pathway[66]. Specifically, LOX inhibitors (NDGA and Rev5901) decreased Bcl-2 and Mcl-1 and increased Bax expression in human pancreatic cancer cells[66]. LOX inhibitors also induced cytochrome-c release and caspase-9 activation. The effect of the LOX inhibitors was also demonstrated *in vivo* where it blocked pancreatic cancer cell growth and induced apoptosis in athymic mice[66]. These studies suggest the relationship between 5-LOX and its role in apoptosis in the tumor microenvironment.

LTB₄ AND PANCREATIC CANCER

LTB$_4$ is a metabolite of 5-LOX and an important inflammatory mediator. LTB$_4$ is involved in recruiting inflammatory cells and is a potent chemokine for monocytes, neutrophils, and eosinophils. It also enhances adhesion and migration of neutrophils across the vascular endothelium[67]. BLT$_1$ and BLT$_2$ are two G-protein-coupled receptors that have a high and low affinity, respectively, for LTB$_4$[68]. LTB$_4$ is secreted from human pancreatic cancer cells and its receptors are upregulated in pancreatic cancer tissue as well as in multiple cell lines[35,69]. Similar to COX-2 and 5-LOX, BLT$_1$ and BLT$_2$ have also been found to be upregulated in PanIN lesions which suggests a potential role of LTB$_4$ and its receptors in chemoprevention[70].

Multiple LTB$_4$ receptor antagonists have been developed but earlier compounds had poor oral bioavailability[68]. A more stable and orally bioavailable compound was later developed, LY293111, which blocks LTB$_4$-mediated kinase phosphorylation[67]. LY293111 inhibits pancreatic cancer growth *in vivo* and *in vitro* through inhibition of proliferation and induction of apoptosis in a variety of pancreatic cancer cell lines (MiaPaCa-2, HPAC, Capan-1, Capan-2, PANC-1, and AsPC-1) in a time- and concentration-dependent manner[69,71]. When LTB$_4$ was added to the cancer cell lines, it stimulated proliferation and induced ERK1/2 phosphorylation in all six cell lines[69]. In a different study, LY293111 was found to cause cell cycle arrest in S phase and suppress cyclin A, cyclin E, and cdk2 expression[71]. When LY293111 was administered to athymic mice with human pancreatic cancer xenografts, the LTB$_4$ receptor antagonist suppressed growth of the subcutaneous xenografts[69].

CLINICAL CORRELATION

COX inhibitors

Multiple studies have been conducted evaluating the use of COX-2 inhibitors combined with different chemotherapy regimens. A phase II trial of Uracil/Tegafur plus Leucovorin and Celecoxib combined with radiotherapy in patients with locally advanced pancreatic cancer did not show a significant response and resulted in substantial gastrointestinal toxicity[72]. A study of Celecoxib and 5-fluorouracil in patients with advanced pancreatic cancer who had progressed after gemcitabine-based chemotherapy showed promising results in that the Celecoxib was well tolerated and capable of inducing durable responses[73]. In a phase II trial of gemcitabine, Irinotecan, and Celecoxib in patients with inoperable pancreatic cancer, the addition of Celecoxib was found to increase the percentage of patients achieving a one-year overall survival from about 3 mo to 9 mo and increased overall survival from about 6 mo to 18 mo[74]. Other studies in patients with advanced pancreatic cancer evaluated the combination of Celecoxib and gemcitabine or the combination of Gemcitabine, Celecoxib, and Cisplatin, but Celecoxib did not increase the efficacy of either chemotherapy regimen (Table 1)[75,76]. While the idea of using a COX-2 inhibitor is promising in patients with pancreatic cancer, it will likely be most effective as a preventive agent very early in the disease process as opposed to improving survival in those patients with advanced disease.

LOX inhibitors

Zileuton is a 5-LOX inhibitor of the N-hydroxyurea series, approved by the Food and Drug Administration in 1996 for the treatment of asthma[25]. It was shown in clinical trials to produce moderate airway improvement in asthmatics. While Zileuton has had promising effects for airway disease, this drug has not yet been tested in patients with cancer.

Several studies have investigated Zileuton in animal

Drug	Type	Trial	Type	Cancer	Outcome	Toxicity
Celecoxib	COX-2 inhibitor	Uracil/Tegafur, Leucovorin, Celecoxib + RT[72]	II	Pancreatic; locally advanced unresectable	No significant partial or complete response	Significant GI toxicity
		Celecoxib, 5-FU[73]	Pilot study	Pancreatic; advanced after Gemcitabine treatment	Durable response	Well tolerated
		Gemcitabine, irinotecan, celecoxib[74]	II	Pancreatic; unresectable	Increased OS from 6 m to 18 m	Well tolerated
		Gemcitabine, celecoxib[76]	II	Pancreatic; locally advanced or metastatic	No significant response	Well tolerated
		Gemcitabine, cisplatin, celecoxib[75]	II	Pancreatic; metastatic	No significant response	Well tolerated
LY293111	LTB4 receptor antagonist	Irinotecan, LY293111[80]	I	Solid tumors (including pancreatic); locally advanced or metastatic	No significant response	Significant GI toxicity
		Gemcitabine, LY293111[81]	II	Pancreatic; locally advanced or metastatic	No significant response	Significant GI toxicity

Cox: Cyclooxygenase; LTB4: Leukotriene B4; RT: Radiation therapy; FU: Fluorouracil; OS: Overall survival; GI: Gastrointestinal.

studies and shown promising results for multiple cancers including carcinoma of the colon, lung, and pancreas. Zileuton was shown to reduce cell proliferation in murine colon adenocarcinoma cell lines[77]. In a xenograft model using human colon cancer cells, Zileuton inhibited tumor growth and reduced tumor mass[78]. In pancreatic cancer studies using the Syrian hamster model with BOP-induced pancreatic cancer, Zyflo (an extended release formulation of Zileuton) was found to reduce the incidence and size of the pancreatic cancer both alone and in combination with a COX-2 inhibitor[79].

LTB4 RECEPTOR ANTAGONIST

A few clinical trials have been conducted using LY293111 in patients with pancreatic cancer. A phase I study demonstrated that LY29311 was well tolerated in combination with Irinotecan although no responses were seen[80]. A different study randomized patients with pancreatic cancer to gemcitabine and LY293111 *vs* gemcitabine and placebo. There was no significant difference in six-month survival or progression-free survival[81]. Finally, a study conducted in patients with non-small cell lung cancer receiving LY293111 and Cisplatin/Gemcitabine also did not show a survival benefit[82]. Similar to COX-2 inhibitors, an LTB4 receptor antagonist would probably be most efficacious early in the disease process.

FLAP inhibitors
MK-886 is a FLAP inhibitor and inhibits leukotriene biosynthesis. It was first developed for use in asthma although clinical development was halted due to only a 50% inhibition of leukotriene production when used[83]. A second-generation FLAP inhibitor, MK-0591, had more potent inhibitory effects on leukotriene production, although it did not clinically perform as expected and was also discontinued[84].

Similar to Zileuton, MK-886 has shown promising results *in vitro* and *in vivo*. As mentioned above, MK-866 was shown to promote growth inhibition in a pancreatic

cancer cell line. It was also shown *in vivo* to reduce pancreatic cancer development in a hamster model[85].

CONCLUSION
The inflammatory pathway is an important process in cancer progression. A combination of clinical studies, epidemiological studies, and basic science investigations indicate that there is a relationship between inflammatory changes in the pancreas and neoplastic progression. Intake of ω-6 polyunsaturated fatty acids provides increased substrate for COX and LOX mediated metabolism of arachidonic acid into eicosanoids. These eicosanoids directly contribute to pancreatic cancer cell proliferation. When COX-2 and 5-LOX are blocked *via* a variety of mechanisms, cancer cell proliferation is abrogated both *in vitro* and *in vivo*. The use of COX-2 and 5-LOX inhibitors in clinical studies in patients with pancreatic cancer has been limited. Patient enrollment has been restricted to patients with advanced disease which makes evaluation of these drugs as chemopreventive agents difficult. COX and LOX expression have been shown to be present during the early neoplastic changes of pancreatic cancer, well before progression to invasive disease. This indicates that the ideal role for these interventions is early in the disease process as preventive agents, perhaps in patients with chronic pancreatitis or hereditary pancreatitis. Further investigation is needed to broaden our understanding of the complex relationship between inflammation and pancreatic cancer and how these inflammatory pathways can be targeted to treat this deadly disease.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Borderline resectable pancreatic cancer: Definitions and management

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Abstract

Pancreatic cancer is the fourth leading cause of cancer death in the United States. While surgical resection remains the only curative option, more than 80% of patients present with unresectable disease. Unfortunately, even among those who undergo resection, the reported median survival is 15-23 mo, with a 5-year survival of approximately 20%. Disappointingly, over the past several decades, despite improvements in diagnostic imaging, surgical technique and chemotherapeutic options, only modest improvements in survival have been realized. Nevertheless, it remains clear that surgical resection is a prerequisite for achieving long-term survival and cure. There is now emerging consensus that a subgroup of patients, previously considered poor candidates for resection because of the relationship of their primary tumor to surrounding vasculature, may benefit from resection, particularly when preceded by neoadjuvant therapy. This stage of disease, termed borderline resectable pancreatic cancer, has become of increasing interest and is now the focus of a multi-institutional clinical trial. Here we outline the history, progress, current treatment recommendations, and future directions for research in borderline resectable pancreatic cancer.

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Key words: Pancreatic cancer; Borderline resectable pancreatic cancer; Neoadjuvant; Vascular resection; Pancreaticoduodenectomy; Whipple

Core tip: Borderline resectable pancreatic cancer has become recognized as a clinical entity worthy of study based on a number of clinical observations that recognize a continuum between resectable and locally advanced unresectable disease. There are few prospective trials and therefore no data to support specific treatment regimens in borderline resectable pancreatic ductal adenocarcinoma (PDAC). Difficulties in achieving a consensus, objective definition, small numbers of patients and variability in therapeutic algorithms have delayed progress in establishing strong evidence-based practices for diagnosis and treatment. The Alliance trial represents a first step in establishing reproducible standards by which future trials in borderline resectable PDAC can abide.

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer death in the United States[1]. While surgical resection remains the only curative option, more than 80% of patients present with unresectable disease[1,2]. Unfortunately, even among those who undergo resection, the reported median survival is 15-23 mo, with a 5-year survival of ap-

proximately 20%[3-5]. Disappointingly, over the past several decades, despite improvements in diagnostic imaging, surgical technique and chemotherapeutic options, only modest improvements in survival have been realized. Nevertheless, it remains clear that surgical resection is a prerequisite for achieving long-term survival and cure. There is now emerging consensus that a subgroup of patients, previously considered poor candidates for resection because of the relationship of their primary tumor to surrounding vasculature, may benefit from resection, particularly when preceded by neoadjuvant therapy.

This stage of disease, termed borderline resectable pancreatic cancer, has become of increasing interest and is now the focus of a multi-institutional clinical trial. Here we outline the history, progress, current treatment recommendations, and future directions for research in borderline resectable pancreatic cancer.

EVOLUTION OF THE BORDERLINE RESECTABLE CONCEPT

The concept of borderline resectable pancreatic cancer has evolved from several clinical observations made over decades. It has been recognized for some time that the prognosis for patients undergoing surgical resection for pancreatic ductal adenocarcinoma (PDAC) is highly dependent on margin status, with total gross excision and histologically negative margins (R0 resection) being associated with the best outcomes. Survival for patients who undergo total gross excision but have histologically positive margins (R1 resection) have a reduced survival in most series[3,6-9]. Most significantly, patient who undergo resection with residual gross tumor (R2 resection) have a prognosis similar to patients treated with non-operative therapy[9-12]. Historically, resectability of pancreatic cancer was defined by absence of distant metastases, absence of local tumor extension to the celiac axis and hepatic artery, as well as the lack of involvement of the superior mesenteric vasculature. However, data emerging in the 1990's suggested that vein resection with negative margins was associated with equivalent survival to standard PD, leading to an increasing acceptance of vascular resection (VR) in curative resections. In 1994, Allema *et al*[13] published a series of 20 superior mesenteric vein/portal vein (SMV/PV) resections, showing no significant differences in survival in comparison to standard PD and confirming both the feasibility of the procedure and the capacity to obtain R0 resections with this technique. In a similarly sized study, Fuhrman *et al*[14] confirmed the findings, concluding that vascular resection is a safe and effective means by which to attain complete resection in cases of tumor adherence to the SMV or SMV/PV confluence. In the ensuing years, others strengthened the notion that appropriately selected patients could undergo vascular resection to achieve survival outcomes similar to patients undergoing standard PD and superior to outcomes of locally advanced disease treated non-operatively[15,16]. In 2004, a group from MD Anderson reviewed all patients who

underwent PD at their institution between 1990 and 2002 to examine the effect of vascular resection on margin status and survival in PDAC[16]. Of 291 patients who underwent PD for PDAC, 181 had a standard PD and 110 had PD with vascular resection. Median survival was 26.5 mo in the standard PD group and 23.4 mo in the group that required VR ($P = 0.18$). Clearly, the extent of venous involvement has a direct relationship to operability and to final margin status. As tumors encroach on the left side of the SMV-portal vein, they encroach increasingly on the SMA. Lu *et al*[17] reported that tumor involvement of greater than half the circumference was highly specific for unresectable disease. The Ishikawa classification, established by Ishikawa *et al* in 1992, is based on radiographic findings that demonstrate the relationship of the tumor to the SMV-PV (1) normal; (2) smooth shift without narrowing; (3) unilateral narrowing; (4) bilateral narrowing; and (5) bilateral narrowing and the presence of collateral veins (Figure 1). This classification has also been used to report the relationship between SMV-PV appearance by cross-sectional imaging and prognosis.

In the early 1990s a small study was conducted in which 28 patients with localized PDAC underwent treatment with preoperative chemoradiation with 5-Fluorouracil (5-FU). After restaging, 17 out of 28 were able to undergo successful resection with few complications, confirming the feasibility and safety of neoadjuvant therapy followed by resection[18]. Similarly, a 1997 study comparing pre-operative and post-operative chemoradiation in 142 patients with resectable disease found pre-operative chemoradiation offered comparable benefits to post-operative therapy and is not hindered by post-operative complications or prolonged recovery[19]. Pisters *et al*[20] found additional advantages of neoadjuvant chemoradiation with 5-FU in 35 patients with resectable PDAC. Among 20 patients who underwent resection, median survival was 25 mo, while median survival among 15 patients who did not undergo PD was 7 mo. They concluded that neoadjuvant chemoradiation results in minimal toxicity while maximizing the number of patients who get combined modality treatment and limiting PD to those most likely to benefit.

Several studies have suggested that neoadjuvant chemoradiation may enhance resectability and inhibit local recurrence[19,21]. A Phase Ⅱ trial published in 1993 demonstrated a significant reduction in the incidence of positive margins and lymph nodes in tumors treated with pre-operative chemoradiation[21]. The authors concluded that negative margin resections achieved in all 10 resected patients, and the low rate of nodal metastasis (10%) may be attributable to neoadjuvant treatment.

Studies of patients with more advanced disease have also proposed that neoadjuvant therapy may result in downstaging, thereby improving the likelihood of R0 resection. In 1999 White *et al*[22] performed a study of 25 patients with locally advanced pancreatic cancer treated with neoadjuvant chemoradiation at Duke University finding that only a small percent were downstaged. 22

Table 1 Comparison of radiographic differences in common definitions for borderline resectable pancreatic cancer			
Effected vessel	AHPBA/SSAT/SSO/NCCN[29]	MD Anderson[28]	Alliance[26]
SMV/PV	Abutment, impingement, encasement of the SMV/PV or short segment venous occlusion	Occlusion	Tumor-vessel interface ⩾ 180° of vessel wall circumference, and/or reconstructable occlusion
SMA	Abutment	Abutment	Tumor-vessel interface < 180° of vessel wall circumference
HA	Abutment or short segment encasement	Abutment or short segment encasement	Reconstructable short segment interface of any degree between tumor and vessel wall
CA	Uninvolved	Abutment	Tumor-vessel interface < 180° of vessel wall circumference

AHPBA/SSAT/SSO/NCCN: Americas Hepatopancreaticobiliary Association/Society for Surgery of the Alimentary Tract/Society of Surgical Oncology/National Comprehensive Cancer Network; SMV/PV: Superior mesenteric vein/portal vein; SMA: Superior mesenteric artery; HA: Hepatic artery; CA: Celiac artery.

of 25 patients underwent restaging after chemoradiation, six of 22 (27.3%) had a decrease in size of the primary tumor and three of the 22 (13.6%) had overall disease regression by radiographic imaging. White *et al*[23] later reported on 111 patients with PDAC, 53 with potentially resectable and 58 with locally advanced disease who underwent neoadjuvant treatment with chemoradiation followed by restaging and surgery as deemed about 11 of 58 (19%) patients with locally advanced disease underwent resection. six of fifty-eight (11%) tumors were radiographically downstaged from locally advanced to potentially resectable by neoadjuvant. Similarly, a slightly larger study at Memorial Sloan-Kettering published in 2001 reported only 3 of 87 (3.4%) patients with locally advanced disease who received neoadjuvant therapy had significant enough responses to warrant surgical exploration[24]. Together, these studies indicate that a small, but real population exists, in which neoadjuvant therapy appears to downstage pancreatic cancer. However, the lack of sensitivity of radiographic staging of pancreatic adenocarcinoma after chemoradiation indicates that radiographic tumor downstaging may not accurately reflect the benefit of neoadjuvant therapy.

Instead, margin status and histologic response may offer more reliable evidence of the efficacy of neoadjuvant therapy. In the above-mentioned studies published by White *et al*[22] in 1999, five of eight patients with either stable disease or disease regression at the time of restaging who underwent exploration were resected. One (4.5%) was resected with negative margins and negative nodes (R0). A later study by the same group reported on 103 patients with potentially resectable or locally advanced disease that underwent neoadjuvant therapy followed by re-staging computed tomography (CT). Of 49 with locally advanced tumors on restaging CT, 11 (22%), were resected, and 6 (55%) of these were resected with negative margins, suggesting that reliance on the standard CT criteria for unresectability will deprive approximately 6 of 49 or 12% of patient of the opportunity for curative (R0) resection after neoadjuvant therapy[25].

Thus, a series of clinical observations lead to the concept of borderline resectable disease. These were well-summarized by Katz *et al*[26]; (1) complete resection of the primary tumor and regional lymph nodes is mandatory for long-term survival; (2) the incidence of margin-negative resection following surgery de novo decreases with increasing involvement of the superior mesenteric vein-portal vein (SMV/PV) and superior mesenteric artery (SMA); (3) resection of the SMV/PV and hepatic artery-but not the SMA-at pancreatectomy is associated with acceptable outcomes; (4) actual tumor regression, so called, "down-staging" of locally advanced cancers is rare following the administration of conventional cytotoxic agents alone or in combination with chemoradiation therapy; and (5) chemotherapy and/or chemoradiation may be used to select patients with favorable tumor biology and physiology who may benefit from aggressive operations.

DEFINITIONS

In general, borderline resectable pancreatic cancer is neither clearly resectable nor clearly unresectable but rather implies a greater chance of incomplete resection in the setting of upfront surgery. Many groups have proposed definitions, however there is not yet a universally accepted definition of borderline resectable pancreatic cancer.

The first published definitions were by the National Comprehensive Cancer Network (NCCN) and the MD Anderson Cancer Center[27,28] (Table 1). In 2009, a consensus statement issued by The Americas Hepatopancreatobiliary Association (AHPBA)/Society for Surgery of the Alimentary Tract (SSAT)/Society of Surgical Oncology (SSO), put forth a third definition, which was later adopted by the NCCN[29]. According to the AHPBA/SSAT/SSO/NCCN definition, borderline resectable PDAC includes tumors that display; (1) venous involvement of the SMV/PV demonstrating tumor abutment, encasement, or short segment venous occlusion, but with suitable vessel proximal and distal to the area of vessel involvement, allowing for safe resection and reconstruction; (2) gastroduodenal artery encasement up to the hepatic artery and short segment encasement/direct tumor abutment of the hepatic artery with no extension to the celiac axis; or (3) tumor-SMA involvement < 180°. This differs from the definition advocated by the M. D. Anderson Group, which is largely similar to the AHPBA/SSAT/SSO/NCCN, except it excludes tumors that abut (< 180° tumor-vessel interface) or encase (⩾ 180° interface) the SMV/PV, instead considering them resect-

able. More recently, Tran Cao *et al*[30] have employed a simplified radiographic classification system-Tumor-vein circumferential interface (TVI)-grouping findings as: no interface, ≤ 180° of vessel circumference, > 180° of vessel circumference, or occlusion. The TVI system was found to be predictive of the need for venous resection, histologic venous invasion, and survival

Additionally, the MD Anderson group has also described two other patient populations, termed borderline resectable "B" and "C" based on clinical, rather than anatomic criteria: those with findings that are suggestive, but not diagnostic of metastasis and patients with marginal performance status[31]. Katz groups B and C were established to recognize clinical subgroups, in addition to the well-recognized anatomic subgroup (Katz Group A), in which staging and treatment for pancreatic cancer were unclear. Many authors acknowledge these clinical definitions, however, few have utilized Katz groups in defining study populations[32-34]. Staging and treatment in clinically defined borderline resectable disease (Groups B and C) deserves attention, however, current efforts focusing on the more widely accepted anatomic definitions have tended to take precedence.

STAGING CONSIDERATIONS IN BORDERLINE RESECTABLE PANCREATIC CANCER

Preoperative evaluation

Preoperative imaging: Optimal outcomes in management of pancreatic cancer require multidisciplinary care, utilizing information from high quality imaging. CT is the most-well studied imaging modality for the evaluation of pancreatic cancer[17,29,35]. Moreover, CT is widely available and familiar to surgeons, making it an optimal imaging study for operative planning. CT should be performed using a so-called, pancreas protocol: tri-phasic contrast (non-contrast, arterial, pancreatic parenchymal, portal venous) in thin cross sectional cuts (≤ 3 mm) with multiplanar reconstructions. While CT performed in this manner has an excellent negative predictive value for unresectability, it is not as accurate at predicting resectability[35]. This is, at least in part, due to its lack of sensitivity for identifying small hepatic and peritoneal metastases.

Recently, some studies have suggested an MRI pancreas protocol may be particularly valuable due to more sensitive visualization of sub centimeter tumors/liver metastases, peritoneal carcinomatosis, and subtle signs of vascular infiltration[36,37].

The role of PET/CT in the evaluation of potentially resectable pancreatic cancer remains unclear. To date, its suggested uses include detection of metastases in high-risk patients, improved diagnostic accuracy for purposes of operative selection and assessment of response to chemoradiation[38-40]. While PET/CT may prove useful in certain circumstances, at this time, pending additional data, its routine use cannot be recommended.

Tissue diagnosis: While histologic diagnosis is not required for patients with presumed pancreatic cancer who are going to be treated with upfront surgery, biopsy is required prior to initiation of neoadjuvant therapy in patients with borderline resectable pancreatic cancer. Fine needle aspiration (FNA) is the preferred method for obtaining a tissue diagnosis. While this can be performed percutaneously, under ultrasound (US) or CT guidance[41], endoscopic ultrasound (EUS) with FNA is favored. Numerous studies have shown that EUS-guided FNA is a safe and cost effective means of increasing diagnostic accuracy in pancreatic cancer[42-44]. Major complications are rare with approximately 2% of patients requiring post-procedure hospitalization[45]. Additionally, EUS-FNA offers decreased potential for peritoneal seeding compared to percutaneous biopsy[46].

In cases where EUS-FNA is not possible, other mechanisms for obtaining a tissue diagnosis may suffice. Intraductal biopsy or brushings may be collected *via* ERCP[47]. This method is particularly useful in borderline resectable pancreatic cancer patients with obstructive jaundice, as these patients should be stented prior to starting neoadjuvant therapy[48,49]. Stenting these patients provides symptomatic relief, reduces risk of cholangitis, prevents coagulopathy, and normalizes LFTs - a requirement in cases where abnormal liver function might result in adverse effects on the metabolism of chemotherapeutics. In the setting of neoadjuvant therapy, expandable short metal stents are preferred as they have longer patency, and therefore are associated with a lower risk of stent occlusion and resultant complication during induction therapy[50,51]. Additionally, covered stents are associated with decreased tumor ingrowth and improved patency and are therefore preferred to uncovered stents[52,53].

Role of CA 19-9: Among many tumor antigens that have been associated with pancreatic cancer, CA 19-9 is the best validated. It is a sialylated Lewis antigen and therefore is not detectable in Lewis antigen negative individuals[54]. Unfortunately, while relatively sensitive, its specificity is suboptimal as CA19-9 levels are often elevated in association with other pancreatic and hepatobiliary pathology, obstructive jaundice in particular[55]. Still, preoperative CA 19-9 has been shown to correlate with pancreatic cancer staging and therefore, resectability[56,57]. Furthermore, post-resection CA 19-9 levels prior to initiation of adjuvant chemotherapy have been shown to have independent prognostic value and can be followed to indicate response to therapy[58-60]. As such, CA 19-9 levels should typically be drawn prior to surgery, following surgery prior to adjuvant therapy and during active surveillance.

Staging laparoscopy: Though there is no absolute consensus on its use, numerous studies have demonstrated that staging laparoscopy can detect occult metastasis

even in pancreatic cancer patients who have undergone high quality cross-sectional imaging[61,62]. Detection of occult metastatic disease such as peritoneal, capsular, or serosal implants, avoids the morbidity associated with laparotomy[63]. In some institutions staging laparoscopy is routine, however others use it selectively in patients with high risk features for advanced disease such as significant weight loss, elevated CA19-9, and borderline resectable disease[56,64,65]. It is reasonable to consider laparoscopy before administering radiation therapy, as it is unlikely that local therapy would confer benefit to patients in the setting of metastatic disease.

Vascular resection: The increasing safety and feasibility of aggressive surgical resections have been central to the evolution of the concept of borderline resectable pancreatic cancer. Still, vascular resection in PD remains an area of controversy. Several studies confirming similar outcomes after PD with SMV-PV resection in comparison to PD alone were crucial in the advent of borderline resectable disease[14,15,66,67]. Even so, two recent, large database studies have called these data into question. In 2012 Castleberry et al[68] published a study using the National Surgical Quality Improvement Program database to analyze all patients undergoing PD. They found that PD with VR was associated with significantly increased morbidity and mortality. Similarly, Worni et al[69] used the National Inpatient Sample database to show comparable increases in morbidity and mortality associated with the addition of VR to PD. These studies are subject to the criticisms of any large database study. In particular, they cannot distinguish the operations performed in which vascular resection was anticipated and planned as opposed to the vascular resection performed in the setting of vascular injury when an adherent tumor is attempted to be removed. These no doubt result in much different rates of blood loss, and morbidity. Nevertheless, these studies call attention to the continued risks associated with vascular resection and are a reminder to emphasize multidisciplinary treatment and planning prior to proceeding with surgical resection in order to reduce perioperative risk in these patients[70].

Data with regard to arterial resection (AR) are even fewer. Some groups suggest similar morbidity and mortality in PD with AR in comparison to PD alone[71,72]. However, most studies indicate that AR significantly increases morbidity and mortality and therefore recommend this approach only for the purposes of obtaining an R0 resection[73]. Additionally, some suggest that AR may provide improved survival in comparison to palliation alone[74-76].

Though not unanimously employed, SMV-PV resection is more widely accepted than AR. In either case, patient selection is paramount to achieving favorable outcomes.

TREATMENT

Despite a paucity of prospective data to support a stan-

dard treatment regimen for borderline resectable pancreatic cancer, neoadjuvant therapy is currently the preferred initial approach[77-79]. Theoretical advantages to neoadjuvant treatment include early treatment of micrometastasis, improved patient selection for surgical intervention, more effective treatment delivery, as well as the potential to achieve some degree of downstaging and/or increase the likelihood of R0 resection. In addition to providing the opportunity to treat early occult disease, neoadjuvant therapy ensures that patients undergoing resection receive multimodality therapy[80]. This is an important benefit as up to 25% of patients with resectable tumors are unable to receive post-operative therapy due to post-operative complications, prolonged recovery or deconditioning[19]. Patients with borderline resectable disease often require more complex resections and it is therefore reasonable to assume delays to receipt of adjuvant therapy may be even more significant. By identifying patients with adequate performance status to complete pre-operative chemotherapy, and tumors with more favorable biology, neoadjuvant therapy selects patients most likely to benefit from resection[81]. In principle, pre-operative treatment may also enable enhanced tumor oxygenation and drug delivery compared to the post operative state, which may result in more effective radiotherapy[82].

In 2001, Mehta et al[83] described the first prospective case series of 15 patients with "marginally resectable" PDAC as indicated by CT evidence of portal vein, superior mesenteric vein, or artery involvement. Patients were treated with 5-FU and radiation followed by reevaluation for resection. Nine of 15 patients underwent resection, all with uninvolved margins, leading the group to conclude that chemoradiation is well tolerated, and may downstage tumors, sterilize regional lymph nodes, and improve resectability in patients with "marginally resectable" pancreatic cancer.

Landry et al[84] reported the first multi-institutional prospective study in borderline resectable PDAC, a randomized phase II trial comparing neoadjuvant regimens. From 2003 to 2005, 21 patients were identified at 10 Eastern Cooperative Oncology Groups institutions. In Arm A, 10 patients, received gemcitabine based chemoradiation, in Arm B 11 patients received induction chemotherapy using gemcitabine/cisplatin/5-FU followed by chemoradiation with 5-FU. 3 patients in Arm A and 2 patients in Arm B were resected. The median survival of resected patients was 26.3 mo. All patients received adjuvant gemcitabine for 5 cycles. The trial was terminated early due to poor accrual, however it found both neoadjuvant regimens to be tolerable, with similar resectability and survival to those reported in retrospective studies.

Aside from these prospective trials, the literature in borderline resectable pancreatic cancer consists mainly of retrospective single institution studies (Table 2).

The first report from MD Ander Cancer Center was a, retrospective review of 160 patients, divided into 3 groups defined by both anatomic and non-anatomic variables[31]. Among these included 84 patients with anatomically defined borderline resectable tumors. Patients

Table 2 Largest studies in borderline resectable pancreatic cancer

Author	Year	Study type	Study size	Number with borderline resectable (definition)	Neoadjuvant	Resected	Negative margins	Median OS (mo)
Chuong *et al*[86]	2013	Single institution retrospective	73	57 (NCCN)	Majority gemcitabine based induction chemotherapy, SBRT	56%	96%	16.4
Katz *et al*[87]	2012	Single institution retrospective	129	115 (AHPBA/SSAT/SSO/ NCCN) or 72 (MDA)	Gemcitabine based chemotherapy and chemoradiation or chemoradiation alone	84% or 78%	95%[1]	33[1]
Barugola *et al*[91]	2012	Single institution retrospective	362	27 (other)	Gemcitabine based chemotherapy and chemoradiation or chemotherapy alone	NR	NR	NR
Kang *et al*[93]	2012	Single institution retrospective	202	35 (NCCN)	Gemcitabine based chemoradiation	91%	87%	26.3
Stokes *et al*[81]	2011	Single institution retrospective	170	40 (MDA)	Capecitabine-based Chemoradiation	46%	75%	23
Chun *et al*[78]	2010	Single institution retrospective	109	109 (other) 74 received neoadjuvant[2]	5-FU or gemcitabine based chemoradiation	100%	59%[2]	23[2]
McClaine *et al*[103]	2010	Single institution retrospective	29	29 (MDA+NCCN hybrid)	Gemcitabine based chemotherapy, chemoradiation or both	46%	67%	23.3
Landry *et al*[84]	2010	Randomized Phase Ⅱ trial	21	21 (other)	Gemcitabine based	24%	60%	26.3
Turrini *et al*[89]	2009	Single institution retrospective	64	49 (MDA)	5-FU/cisplatin based chemoradiation	18%	100%	24
Katz *et al*[31]	2008	Single institution retrospective	160	160 (MDA)	Gemcitabine based chemotherapy, chemoradiation	41%	94%	40

[1]Results for Americas Hepatopancreaticobiliary Association/Society for Surgery of the Alimentary Tract/Society of Surgical Oncology/National Comprehensive Cancer Network (AHPBA/SSAT/SSO/NCCN) definition of borderline resectable; [2]Results for patients who received neoadjuvant treatment. NR: Not reported.

were treated with a variety of neoadjuvant regimens incorporating chemotherapy, chemoradiation, or both, prior to planned resection. Of this group, 38% underwent resection - 97% of which were R0. The median survival of all patients was 21 mo: 40 mo for resected patients and 15 mo for patients who did not undergo resection. Since this study, multiple smaller and few similarly sized retrospective reviews have reported similar findings.

Small *et al*[85] first used the NCCN definition of borderline resectable disease in a multi center, phase Ⅱ trial of lesser degree, enrolling 41 patients, including 9 with borderline resectable disease. The study used neoadjuvant full dose gemcitabine plus radiation therapy, and found that treatment was well tolerated and that 33% of were able to proceed with resection. They observed a 76% one-year survival rates, and concluded that the strategy should be further explored.

Numerous other small-scale studies demonstrate the safety and efficacy of other neoadjuvant regimens. Stokes *et al*[81] performed a retrospective review of 170 cases of PDAC and identified 40 cases of borderline resectable pancreatic cancer according to the M.D. Anderson definition (A: 30; B: 5; C: 5)[31]. These patients underwent accelerated chronomodulated capecitabine-based chemoradiation using stereotactic-based radiotherapy. About 34 of 40 (85%) borderline resectable patients completed neoadjuvant therapy and were restaged, 16 (46%) of these underwent successful resection. R0 resection rate among these patients was 75%. The group concluded that accelerated chronomodulated capecitabine-based chemoradiation with stereotactic-based radiotherapy was

an efficient and well-tolerated treatment. Most recently, Chuong *et al*[86] performed a retrospective review of 73 patients who were treated with induction chemotherapy with Gemzar, Taxotere, and Xeloda and stereotactic body radiation therapy at H. Lee Moffitt Cancer Center. This included 57 patients with borderline resectable disease as designated by the NCCN definition[27]. Among 32 borderline resectable patients who underwent resection, only one patient (3.1%) had an R1 resection, while 31 patients (96.9%) had R0 resections, and median overall survival was 20 mo. It is clear that across studies, approximately one third of patients can go on to successful resection, however, small study size, inconsistent definitions of disease and a multitude of neoadjuvant strategies make it impossible to draw other definitive conclusions from these studies.

The same constraints have also made it difficult to establish anatomic guidelines for decision-making. The Fox Chase group performed a retrospective review of 109 patients with PDAC involving the PV/SMV in an effort to better delineate the degree of involvement of the PV/SMV that best defines the group of patients who would benefit from neoadjuvant therapy and resection (borderline disease)[78]. The patients were grouped according to Ishikawa classification with types Ⅱ and Ⅲ equating to unilateral involvement in 67 patients, while types Ⅳ and Ⅴ were used to describe bilateral involvement in 42 patients. Pre-operative chemotherapy improved resection rates and overall survival in Ishikawa types Ⅱ and Ⅲ (unilateral involvement), but not types Ⅳ and Ⅴ (bilateral involvement). R0 resection

rates in the neoadjuvant and primary resection groups were 71% and 5%, respectively ($P = 0.0001$) for types Ⅱ and Ⅲ, but 41% and 23%, respectively ($P = 0.25$) for types Ⅳ and Ⅴ. Similarly, median overall survival rates with and without neoadjuvant were 26 and 10 mo, respectively ($P = 0.0001$) Ishikawa type Ⅳ and Ⅴ patients, were 21 and 22 mo, respectively ($P = 0.48$). While this study supports the benefit of neoadjuvant therapy in patients with Ishikawa type Ⅱ and Ⅲ *vs* in types Ⅳ and Ⅴ, increased median overall survival in patients who underwent primary resection with types Ⅳ and Ⅴ (22 mo) in comparison to types Ⅱ and Ⅲ (10 mo) highlight the difficulty in drawing accurate conclusions due to small study size.

More recently, Katz *et al*[87] applied Response Evaluation Criteria in Solid Tumors (RECIST) criteria to determine the effect of neoadjuvant therapy on anatomic extent and size reduction in borderline resectable PDAC. They reported on 129 patients with borderline resectable tumors who underwent neoadjuvant treatment at MD Anderson. 122 of them were restaged and of these, only 15 (12%) showed partial response by RECIST criteria. Despite this, 85 (69%) underwent resection, 81 (95%) were R0. Median overall survival of those who underwent resection was 33 mo, which did not correlate with RECIST response indicating that a lack of radiographic evidence of tumor response in PDAC is of little clinical value as prognostic or predictive marker. The authors therefore suggest aggressive surgical resection in patients with adequate performance status and absence of disease progression.

Like the United States, Asia and Europe have tended toward increasingly aggressive treatment of borderline resectable pancreatic cancer. Europeans have focused on chemotherapy rather than radiation therapy, seeking improved neoadjuvant and adjuvant regimens to control systemic disease-as this is the most common cause of treatment failure[11,88-92]. Asian countries have also employed neoadjuvant strategies, but with increased emphasis on determining how it effects surgical resection[93-96]. Additionally, they have focused on defining radiographic criteria to predict surgical outcomes as well as surgical aspect that influence outcomes, such as likelihood of R0 resection, and need for vascular resection[97-100].

Need for standardization

The lack of uniformity in the definition of borderline resectable PDAC has been an obstacle to evaluating the optimal preoperative assessment, therapeutic strategy and surgical decision-making regarding this group of patients[26]. In recognition of a growing national interest in serving patients with borderline resectable PDAC, and to establish an infrastructure in which to acquire data through multi-institutional trials, The Alliance for Clinical Trials in Oncology (Alliance), in cooperation with the Southwest Oncology Group, Eastern Cooperative Oncology Group, and Radiation Therapy Oncology Group, has received support by the NCI to conduct a multi-institutional treatment trial for patients with borderline resectable PDAC (Alliance A021101). This trial was designed as a single arm pilot study with the intent to utilize a standard objective definition based on cross-sectional imaging, and to determine if a there was a sufficient patient population to conduct cooperative group trials. The study design employs a neoadjuvant design with induction chemotherapy and chemoradiation therapy, surgery and adjuvant chemotherapy[26,84].

With an aim to establish a clear, reproducible means by which to define borderline resectable PDAC by radiologic criteria, the trial has recognized any one or more of the following identifiers of borderline resectable PDAC: (1) interface exists between tumor and the SMV/portal vein measuring 180 degrees or greater of the vessel wall circumference, and/or reconstructable venous occlusion; (2) interface exists between tumor and the SMA measuring less than 180 degrees of the vessel wall circumference; (3) a reconstructable, short-segment interface of any degree exists between tumor and the common hepatic artery; and/or (4) interface exists between tumor and the celiac trunk measuring less than 180 degrees of the vessel wall circumference.

Using this definition, the trial will evaluate the survival, outcomes and toxicity rates using 4 cycles of mFOLFIRINOX (oxaliplatin 85 mg/m^2, irinotecan 180 mg/m^2, leucovorin 400 mg/m^2, 5-fluorouracil 2400 mg/m^2) followed by external beam radiation therapy (50.4 Gy) with capecitabine (825 mg/m^2). After re-staging, patients who are deemed candidates for resection proceed with surgery followed by post-operative gemcitabine.

The use of modified FOLFIRINOX (mFOLFIRINOX) as induction therapy in the Alliance Trial is based on the superior survival and response rates observed for FOLFIRINOX in metastatic pancreatic cancer in a randomized controlled trial of 342 patients with metastatic pancreas cancer. The dosing was modified in an attempt to partially circumvent the greater toxicity associated with FOLFIRINOX in comparison to gemcitabine. While FOLFIRINOX displayed improved median overall survival (11.1 mo *vs* 6.8 mo, $P < 0.001$), median progression-free survival (6.4 mo *vs* 3.3 mo, $P < 0.001$) and objective response (31.6% *vs* 9.4%, $P < 0.001$), toxicities including neutropenia, febrile neutropenia, fatigue, vomiting and diarrhea were all worse with FOLFIRINOX[101]. The Alliance Trial is therefore utilizing a modified regimen, or mFOLFIRINOX, in which the 5-FU bolus has been dropped, but all other dosing remains the same, in an effort to reduce these toxicities.

After resection, borderline resectable pancreatic caner is treated similar to any other resected PDAC. Consequently, adjuvant chemotherapy in this trial is administered according to the standard gemcitabine regimen used following resection of PDAC[102].

This benchmark trial will assess the feasibility of multi-institutional efforts to study the subset of patients regarded as having borderline resectable disease and establish a foundation for future studies in this group of
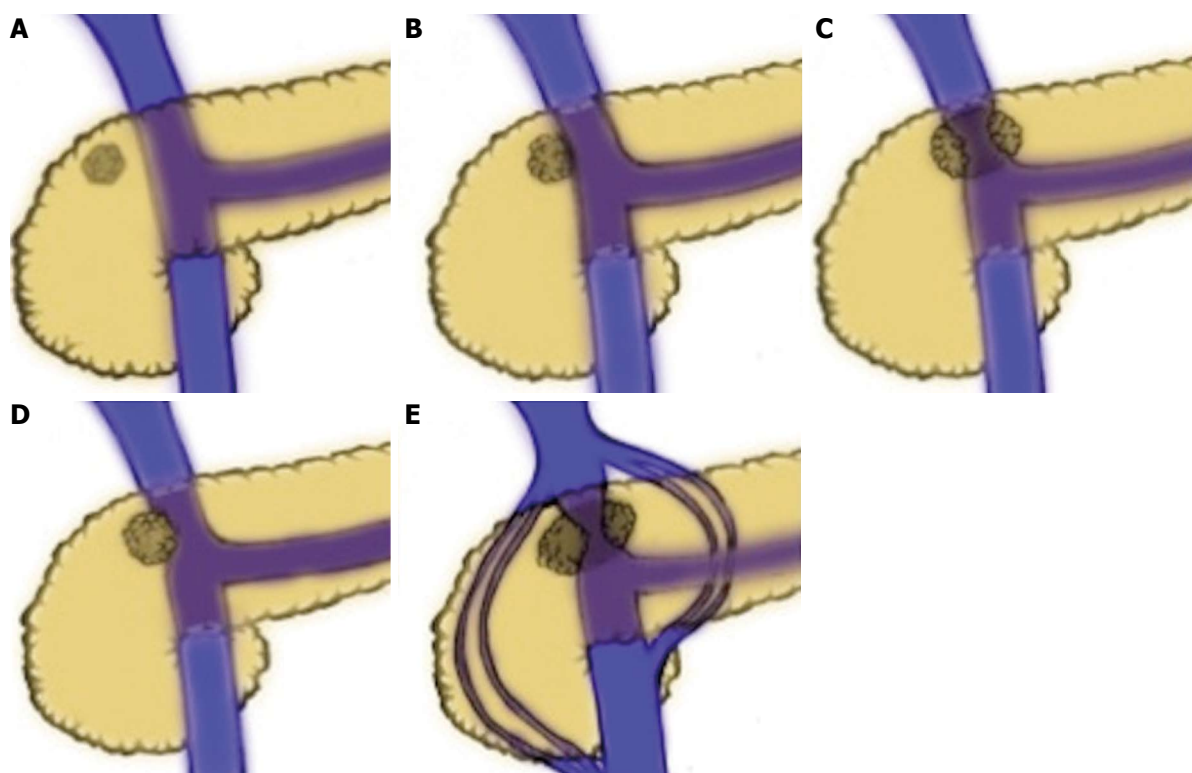
Figure 1 Ishikawa classification of portal and/or superior mesenteric vein involvement. A: Normal; B: Smooth shift without narrowing; C: Unilateral narrowing; D: Bilateral narrowing; E: Bilateral narrowing with collateral veins.

patients. While the primary endpoint of the study is, in fact, accrual, it will be of great interest to assess the activity of the neoadjuvant regimen by secondary endpoints such as the number of patients who undergo negative margin resection and overall survival. As of December 14, 2013, 14 of a targeted 20 patients had been accrued, suggesting a promising outcome for this trial.

CONCLUSION

Borderline resectable pancreatic cancer has become recognized as a clinical entity worthy of study based on a number of clinical observations that recognize a continuum between resectable and locally advanced unresectable disease. There are few prospective trials and therefore no data to support a specific neoadjuvant therapy regimen in borderline resectable PDAC. However, numerous studies suggest that patients with borderline resectable PDAC who receive neoadjuvant therapy can go on to R0 resection and enjoy outcomes similar to disease that is originally resectable[81,88,103]. Taken together the available data suggests that approximately one-third of initially borderline resectable pancreatic tumors may be proceed successful resection following receipt of neoadjuvant therapy[104]. Difficulties in achieving a consensus, objective definition, small numbers of patients and variability in therapeutic algorithms have delayed progress in establishing strong evidence-based practices for diagnosis and treatment. The Alliance trial represents a first step in establishing reproducible standards by which

future trials in borderline resectable PDAC can abide.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Emerging role of the KRAS-PDK1 axis in pancreatic cancer

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Abstract

Pancreatic cancer is a highly aggressive tumour that is very resistant to treatments and it is rarely diagnosed early because of absence of specific symptoms. Therefore, the prognosis for this disease is very poor and it has the grim supremacy in terms of unfavourable survival rates. There have been great advances in survival rates for many types of cancers over the past few decades but hardly any change for pancreatic cancer. Mutations of the Ras oncogene are the most frequent oncogenic alterations in human cancers. The frequency of *KRAS* mutations in pancreatic cancer is around 90%. Given the well-established role of KRAS in cancer it is not surprising that it is one of the most attractive targets for cancer therapy. Nevertheless, during the last thirty years all attempts to target directly KRAS protein have failed. Therefore, it is crucial to identify downstream KRAS effectors in order to develop specific drugs able to counteract activation of this pathway. Among the different signalling pathways activated by oncogenic KRAS, the phosphoinositide 3-Kinase (PI3K) pathway is emerging as one of the most critical KRAS effector. In turn, PI3K activates several parallel pathways making the identification of the precise effectors activated by KRAS/PI3K more difficult. Recent data identify 3-phosphoinositide-dependent protein kinase 1 as a key tumour-initiating event downstream KRAS interaction with PI3K in pancreatic cancer.

Core tip: Recent evidence suggests that protein kinase 1 (PDK1) is a key oncogenic driver in pancreatic cancer. Furthermore, PDK1 appears to be activated downstream the main pancreatic cancer oncogene *KRAS* that is mutated in nearly all pancreatic adenocarcinomas. This evidence suggests that PDK1 could represent a novel target in the treatment of pancreatic cancer.

INTRODUCTION

Pancreatic cancer is a deadly disease both because it is generally discovered very late but also because it is very resistant to chemotherapy and radiation therapy[1]. In addition, pancreatic cancer metastasizes very early and recent data suggest that many patients are likely to harbour metastases at the time of diagnosis[2]. The most common form of pancreatic cancer occurs in the exocrine cells of the pancreas[3]. The exocrine pancreatic tumours account for over 95% of all pancreatic cancers, and can occur anywhere in the pancreas, although most often they are found in the head of the pancreas. Pancreatic ductal adenocarcinoma (PDAC) is the most common type, representing almost 90% of all exocrine tumours.

PDACs develop from cells lining the ducts that carry the digestive juices into the main pancreatic duct and then on into the duodenum. Like other solid tumours, pancreatic cancer is the result of a multistep process. Its initiation and development involves specific genetic changes enabling growth and survival mechanisms, initiation of a marked desmoplastic reaction and finally tissue invasion and metastasis[4]. The signalling pathways regulating tumourigenesis are the result of multiple interactions between the pancreatic cells themselves, the supporting stroma and the immune system[5].

A careful molecular and pathological analysis of evolving PDAC has revealed a characteristic pattern of histologically defined precursors, named pancreatic intraepithelial neoplasia (PanIN), that has been excellently modelled by Hruban and colleagues[6]. In brief, the morphology of the tumour progresses in steps from normal ducts consisting of normal pancreatic duct cells to aberrant ducts with disorganised cell formations and differentiation grade, and finally to infiltrating cancer. These morphological changes occur along with several genetic lesions. A comprehensive genome analysis of 24 human pancreatic cancers revealed an average of 63 genetic alterations[7]. These alterations, mainly point mutations, affect distinct cellular pathways that can be classified in 12 distinct signalling pathways or processes: apoptosis, control of G1/S phase transition, Hedgehog signalling, KRAS signalling, TGF-beta signalling, Wnt/Notch signalling, DNA damage control, homophilic cell adhesion, Integrin signalling, JNK signalling, Invasion and small GTPase signalling (other than KRAS). The first six of these core pathways/processes were found to be genetically altered in all the analysed samples and the last six were altered in 16-23 of the 24 samples[7]. A recent comprehensive evaluation of the pancreatic cancer genome has revealed a multitude of additional mutated genes involved in chromatin modification and genes associated with embryonic regulation of axon guidance[1].

The progression from normal duct epithelium to infiltrating PDAC involves a series of genetic alterations in conjunction with morphological changes. Activating *KRAS* mutation and overexpression of ERBB2 occur early in the progression (PanIN-1), inactivation of the cyclin-dependent kinase inhibitor 2A at an intermediate stage (PanIN-2) and inactivation of TP53, SMAD4 and BRCA2 occur at a late stage (PanIN-3)[1,7].

Activating *KRAS* mutations are the first genetic changes that are detected in the progression from PanIN-1 to PanIN-3, even though sporadic mutation can be found in histologically normal pancreas and in lesions that show the earliest stages of histological alterations. With disease progression, the prevalence of *KRAS* mutation increases and occurs in over 90% of PDACs[1,8-10]. Understandably, KRAS-dependent pathways represent the main target in strategies attempting to counteract pancreatic cancer progression. In this review we will discuss the evidence suggesting that targeting the phosphoinositide 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase 1 (PDK1) pathway can be a valid strategy to counteract KRAS signalling in pancreatic cancer.

KRAS

The small GTPase KRAS is frequently mutated in human cancers, with mutations occurring in nearly all tumours. Activating *KRAS* mutations involve only specific amino acids which interfere with the GTPase activity. Most mutations in pancreatic cancer change a glycine at amino acid 12 to a valine or aspartate (KRAS^{G12V} and KRAS^{G12D} respectively) and have a well-established role in the initiation and progression of PDAC[11,12]. The *KRAS* mutation result in a constitutively active protein that promotes persistent signalling to downstream effectors[13]. In turn, this hyperactivated signalling results in enhanced stimulation of proliferative pathways, thus conferring a growth advantage to the cancer cell. Several genetic studies have shown that activating *KRAS* mutations are necessary for the onset of pancreatic cancer[14]. An inducible pancreas-specific expression system was used recently to show that KRAS^{G12D} expression is also required for tumour maintenance[15]. In addition to cancer, *KRAS* mutations have also been identified in benign conditions such as chronic pancreatitis which result in increased risk of developing PDAC[16]. KRAS signals *via* a number of downstream effectors, amongst others RAF kinase, PI3K, guanine exchange factors for the small GTPases RAL (RAL-GEFs) and phospholipase Cε. In PDAC the main signalling pathways downstream of KRAS are the PI3K pathway and the mitogen-activated protein kinase (MAPK) cascade. Studies in pancreatic duct epithelial cell systems have demonstrated that the transforming potential of oncogenic *KRAS* is dependent on PI3K signalling and mutated KRAS is associated with up-regulation of survival signals including the PI3K/Akt survival pathway[17]. Knock-down of KRAS in pancreatic cancer cells demonstrated reduced activation of several proteins including Akt and ERK, indicating a key role for KRAS in regulation of the PI3K signalling pathway and the MAPK signalling cascade. Members of the MAPK network are rarely genetically modified in pancreatic cancer but this signalling pathway can be hyperactivated by constitutively active KRAS. Indeed targeting the RAF/MEK/ERK pathway in the MAPK cascade with selective drugs has shown promising effects on pancreatic cancer growth. The MAPK cascade and the PI3K pathway are both classically activated *via* Receptor Tyrosine Kinases like the epidermal growth factor receptors (EGFR). Since EGFR gene (*ERBB2*) amplification is one of the early genetic events in the development of pancreatic neoplasia these pathways can be further activated through EGFR in pancreatic cancer[18].

PI3K PATHWAY

The PI3K pathway is involved in inhibition of apoptosis and stimulation of cell proliferation and it has been estimated that at least 50% of all cancer types are related to deregulation of this signalling pathway[19]. Of the 8 mam-

malian PI3K isoforms gain of PIK3CA (PI3K/p110α) function by mutation is common in several human cancers[20,21]. On the other hand we have recently shown that the PI3K isoform p110γ is specifically overexpressed in PDAC[22]. Upon activation PI3Ks catalyse the phosphorylation of phosphoinositides promoting recruitment of downstream signalling molecules such as Akt and PDK1 to the plasma membrane which in turn induce several physiological functions such as cell growth, cell survival, cell migration, and cell cycle entry[23]. This activation is negatively regulated by the tumour suppressor phosphatase and tensin homolog (PTEN)[24]. PTEN mutations are rare in human PDAC, but loss of PTEN function has been shown to be involved in pancreatic cancer resulting in sustained PI3K activation[25]. Furthermore, animal models with KRAS[G12D] activation and PTEN deletion develop pancreatic cancer with an accelerated phenotype of acinar-to-ductal metaplasia, leading to PanIN and cancer progression[26].

Increased activation of the PI3K effector Akt was shown to be a common feature and a biological indicator of aggressiveness in PDAC[27,28]. Additionally, it has been reported that Akt is a regulator of cell plasticity in the pancreas. Indeed it has been shown that constitutively active Akt induced expansion of the ductal compartment, and also led to premalignant lesions *in vivo*[29].

PI3K signalling in the microenvironment has further been demonstrated to enhance tumour progression. Specifically, blocking PI3K/p110γ expressed by myeloid cells in the stroma significantly suppresses tumour growth and invasion[30].

KRAS/PI3K/PDK1 AXIS

It has been recently shown that PDK1 is required for anchorage-independent and xenograft growth of breast cancer cells harbouring either *PI3KCA* or *KRAS* mutations[31]. The most compelling evidence for the existence of a KRAS/PI3K/PDK1 axis derives from a recent study demonstrating that PI3K-PDK1 signalling is an essential node of non-oncogene addiction in KRAS-driven pancreatic cancer initiation and maintenance[32].

Indeed, using genetic and pharmacological approaches KRAS/PI3K/PDK1 axis has been shown to be an essential pathway for pancreatic cancer being able to induce cell plasticity, acinar-to-ductal metaplasia, intraepithelial neoplasia, and pancreatic cancer formation as well as tumour maintenance. Interestingly, the authors further showed that ablation of PDK1 specifically in the epithelial compartment of the lung using two different recombination strategies, had no significant inhibitory effect on KRAS[G12D]-induced Non-small-cell lung carcinoma (NSCLC) development and progression, supporting the conclusion that PDK1 might have a specific role downstream of KRAS in pancreatic cancer. Nevertheless, more evidence is required to conclude that PDK1 has a specific role downstream of KRAS in pancreatic cancer.

On the other hand, this demonstrates that there are substantial tissue- and context-specific differences in activation of KRAS effectors. Such differences may have important clinical implications because they could explain the diverse response to targeted therapies of different tumour types harbouring oncogenic KRAS mutations. Indeed, a recent study showed no substantial response of KRAS[G12D]-driven NSCLC toward PI3K-mTOR inhibition *in vivo*[33]. We have recently reported that the PDK1-specific inhibitor 2-*O*-benzyl-*myo*-inositol 1,3,4,5,6-pentakisphosphate (2-*O*-Bn-IP5), strongly reduced the number of surviving pancreatic cancer cells *in vitro*[34]. Our data further revealed that 2-*O*-Bn-IP5 is able to sensitise cancer cells, including pancreatic cancer cells, to the pro-apoptotic effect of anti-cancer drugs. Our data thus provide further evidence for the rationale to investigate KRAS-driven oncogenic pathways in a tissue- and context-specific manner to characterize the relevant nodes engaged in different tumour entities.

Interestingly, recent work has revealed that PDK1 directly phosphorylates the Polo-like kinase 1 (PLK1) which in turn induces MYC phosphorylation[35]. This novel PDK1-PLK1-MYC signalling regulates cancer cell growth and survival. In addition, it has been shown that MYC controls generation of self-renewing metastatic pancreatic cancer cells[36]. Indeed stable expression of activated KRAS[G12D] confers a large degree of phenotypic plasticity to cells that predisposes them to neoplastic transformation and acquisition of stem cell characteristics. Ischenko *et al*[36] demonstrated that metastatic conversion of KRAS[G12D]-expressing cells, that exhibit different degrees of differentiation and malignancy, can be reconstructed in cell culture, and that the proto-oncogene c-MYC controls the generation of self-renewing metastatic cancer cells. These results provide evidence that the conversion of precancerous to cancerous cells is determined by oncogenic RAS-induced transcription factors, primarily MYC. In addition, a cooperative mechanism between mutant *KRAS* and *PIK3CA* has been recently shown, in part mediated by RAS/p110α binding, as inactivating point mutations within the RAS-binding domain of PIK3CA significantly ablates signalling pathways[37]. Indeed somatic cell knock-in of both KRAS[G12V] and oncogenic PIK3CA mutations in human breast epithelial cells results in cooperative activation of the PI3K and MAPK pathways in vitro, and leads to tumour formation in immunocompromised mice. Xenografts from double knock-in cells retain single copies of mutant *KRAS* and *PIK3CA*, suggesting that tumour formation does not require increased copy number of either oncogene. More importantly PDK1 seems to play a key role in this cooperativity, since PDK1-dependent activation of the downstream effector p90RSK is increased by the combined presence of mutant KRAS and PIK3CA. Finally, PDK1 has been recently found significantly overexpressed in the high-grade intraductal papillary mucinous neoplasms (IPMN) *vs* low-grade IPMN and in pancreatic and intestinal-type of IPMN *vs* gastric-type of IPMN[38]. These data suggest that PDK1 may play a role in development of IPMN invasive cancer.

MIR-375, AN ADDITIONAL LINK BETWEEN KRAS AND PDK1

MicroRNAs (miRNAs) modulate the expression levels of mRNAs and proteins and can contribute to cancer initiation and progression[39]. In addition to their intracellular function, miRNAs are released from cells and shed into the circulation. Increasing interest has been recently focused on the role of miRNAs in pancreatic cancer malignant progression[40]. It has been reported that changes in miRNAs expression patterns during progression of normal tissues to invasive pancreatic adenocarcinoma in the p48-Cre/LSL-KRAS^{G12D} mouse model mirrors the miRNAs changes observed in human pancreatic cancer tissues[41]. It was found that the expressions of miR-148a/b and miR-375 were decreased whereas the levels of miR-10, miR-21, miR-100 and miR-155 were increased in invasive carcinoma compared to normal tissues in the mouse model. Similar data have been found in KRAS oncogene transgenic rats with PDAC[42]. Recently, miR-375 has been found downregulated in different cancers including pancreatic cancer, and suppresses key cancer functions by targeting several signalling molecules such as PDK1[43]. It is worth noting that RAS can up-regulate PDK1 expression. Indeed, it has been shown that RAS drives monocytic lineage commitment in granular monocyte bipotential cells by promoting the expression of PDK1[44]. Interestingly, a recent study investigated the transcriptional regulation of miR-375 validated target PDK1[45] in pancreatic carcinoma[46]. miR-375 was observed to be downregulated in the tumour compared with non-tumour tissues from patients with pancreatic cancer[41]. As determined by a luciferase reporter assay, the ectopic expression of miR-375 was able to reduce the transcriptional activity of PDK1 and the expression of endogenous PDK1 protein levels. Functional assays showed that miR-375 was able to inhibit proliferation and promote apoptosis of pancreatic cancer cells[46]. Therefore, miRNA-375 appears to be a key regulator of PDK1, suggesting that it may have a potential therapeutic role in the treatment of pancreatic cancer. Furthermore, this evidence suggests that miR-375 may represent an additional link between KRAS and PDK1 since KRAS-induced downregulation of miR-375 results in increased PDK1 expression.

CONCLUSION

This review provides evidence for a role of the KRAS/PDK1 axis in pancreatic cancer. Given the fact that KRAS is considered an "undruggable" protein the identification of downstream targets is of value for the future development of alternative pharmacological strategies to block KRAS-dependent signalling pathways. Highly selective PDK1 inhibitors are now available and combination strategies may achieve more effective blockade of this axis. At AACR 2012, a study demonstrated that nanoparticles delivery of a novel AKT/PDK1 inhibitor inhibits pancreatic cancer tumour growth[47]. MicroRNAs may provide alternative strategies for intervention. For instance miR-375 that is downregulated in pancreatic cancer can be used as an alternative strategy to counteract the KRAS/PDK1 axis. Interestingly, miR-375 has been found downregulated in multiple types of cancer, and suppresses core hallmarks of cancer by targeting several important oncogenes such as Yes-associated protein 1 (YAP1), insulin-like growth factor 1 receptor (IGF1R) and PDK1[43]. These oncogenes might play a key role in pancreatic adenocarcinoma progression. For instance, YAP1 has been found overexpressed in pancreatic cancer tissues and might play an important role in pancreatic cancer growth[48]. More importantly, IGF1R is emerging as a novel promising new drug targets in pancreatic cancer therapy[49]. Therefore, the understanding of the role of the KRAS/PDK1 axis in pancreatic cancer might provide a number of novel therapeutic opportunities for a cancer that urgently needs immediate response to counteract its grim reality.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Role of endoscopic ultrasound in the molecular diagnosis of pancreatic cancer

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Abstract

Pancreatic ductal adenocarcinoma remains one of the most deadly types of tumor. Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) is a safe, cost-effective, and accurate technique for evaluating and staging pancreatic tumors. However, EUS-FNA may be inconclusive or doubtful in up to 20% of cases. This review underlines the clinical interest of the molecular analysis of samples obtained by EUS-FNA in assessing diagnosis or prognosis of pancreatic cancer, especially in locally advanced tumors. On EUS-FNA materials DNA, mRNA and miRNA can be extracted, amplified, quantified and subjected to methylation assay. *Kras* mutation assay, improves diagnosis of pancreatic cancer. When facing to clinical and radiological presentations of pseudo-tumorous chronic pancreatitis, wild-type *Kras* is evocative of benignity. Conversely, in front of a pancreatic mass suspected of malignancy, a mutated *Kras* is highly evocative of pancreatic adenocarcinoma. This strategy can reduce false-negative diagnoses, avoids the delay of making decisions and reduces loss of surgical resectability. Similar approaches are conducted using analysis of miRNA expression as well as Mucin or markers of invasion (S100P, S100A6, PLAT or PLAU). Beyond the diagnosis approach, the prediction of response to treatment can be also investigated form biomarkers expression within EUS-FNA materials.

Core tip: This review depicts the widespread potential for the molecular analysis of samples obtained by ultrasound-guided fine needle aspiration in assessing diagnosis or prognosis of pancreatic adenocarcinoma, as well as translational studies on new markers and epigenetic alterations. Among these markers, *Kras* oncogene assay appears now the most robust for improvement of positive and differential diagnosis of pancreatic cancer. Clinical implication of miRNA, Mucins and markers of invasion is still debated. Future molecular developments may open windows towards personalized treatments after molecular characterization of a single patient.

Bournet B, Gayral M, Torrisani J, Selves J, Cordelier P, Buscail L. Role of endoscopic ultrasound in the molecular diagnosis of pancreatic cancer. *World J Gastroenterol* 2014; 20(31): 10758-10768 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i31/10758.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i31.10758

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most deadly types of tumor. The 5-year survival

rate after diagnosis is < 3.5%[1]. The only curative treatment is surgical resection but this surgery is possible in only 10% to 15% of cases. The remaining cases with locally advanced and/or metastatic pancreatic cancer are treated in a palliative way with chemotherapy (Gemcitabine or FOLFIRINOX) or best supportive cares[1]. This dismal prognostic is partly due to the lack of robust markers for the early diagnosis of PDAC that may jeopardize treatment efficacy in a subset of patients. Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) is a rapid, safe, cost-effective, and accurate technique for evaluating and staging pancreatic tumors[2-6]. In addition, EUS-FNA is the main clinical appliance for cytological and histological material collection from locally advanced PDAC that represents 85% of pancreatic cancer patients. However, its accuracy for the diagnosis of malignancy varies widely with a sensitivity ranging from 65% to 95%, and with a mean accuracy of 85% (negative predictive value ranging from 50% to 70%). Despite the miniaturization of histological samples provided by the FNA using 22 Gauge needle, immunohistochemistry can be achieved when micro biopsies are collected, fixed and embedded in paraffin. In our experience, micro-biopsies can be thus obtained in near 80% of cases. These Immunodiagnostic can be useful to differentiate for instance PDAC from endocrine tumors. It is harder to differentiate malignant from inflammatory lesions of exocrine pancreas. Despite modern imaging techniques, difficulties persist to early diagnose PDAC and to differentiate PDAC from benign diseases such as chronic pancreatitis especially in its pseudotumoral form[2-5]. It is indeed critical to avoid unnecessary resection of benign lesions (such as focal lesions of chronic pancreatitis or autoimmune pancreatitis) or to delay the treatment of PDAC in a subset of patients. Finally EUS-FNA may be inconclusive or doubtful in up to 20% of cases[2-7]. An explanation for an inconclusive cytopathology is multiple: (1) in PDAC the presence of desmoplastic reaction often associated with poor cellularity; (2) distinguishing well-differentiated PDAC and reactive atypia is difficult to appreciate in small samples; (3) small tumors are often not easy to biopsy and performances of cytopathology are lower[7]; and (4) well vascularized tumors that have a high risk of coagulating within the FNA materials. In cases where there is an inconclusive biopsy, a doubt persists regarding the presence or not of malignancy. Some technical improvements have been developed such as contrast ultrasound, elastography, new generations of needle (procore biopsy needle), or transport media for samples[8-11]. However, a subset of samples remained inconclusive and accuracy of EUS-FNA is still around 80%-85%. In parallel, the improvement of molecular biology techniques including DNA and RNA amplification permits the analysis and the quantification of molecular markers in cytological samples, especially from EUS-guided FNA of pancreatic lesions[12-17]. In addition, EUS-FNA that allows sampling of biological material and molecular biology is mandatory not only for pathologists but also

for scientists to discover new molecular biomarkers for this disease. This review depicts the widespread potential for the molecular analysis of samples obtained by EUS-FNA in assessing diagnosis or prognosis of PDAC, as well as translational studies on new markers and epigenetic alterations.

POTENTIAL OF MOLECULAR ANALYSIS ON EUS-FNA MATERIALS
DNA extraction
Despite using fine needles, sufficient materials can be obtained for cytology and histology. A portion of this material, collected following air or saline flushing of the needle once the core biopsies have been reclaimed for histopathology, can be used for further molecular analysis. A mean of 550 nanograms of DNA (range 100 nanograms to 1.5 mg) is obtained and DNA amplification is possible in 98 to 100 of cases[18]. For comparison, previous studies and protocols conducted on pure pancreatic juice attested for a lack of extraction/amplification in almost 13% of samples[19-21]. Thereafter, purified DNA authorizes PCR followed by Restriction Fragment Length Polymorphism or sequencing. Recently we developed an allelic discrimination assay on material sampled on EUS-FNA as well as specific Methylation-Specific PCR assay[22]. All these procedures are successful in almost 100% of the cases, in the absence of DNA pre-amplification. This is of prime importance because DNA amplification generates mutations especially when using a low amount of starting material that can eventually bias subsequent analysis. In addition, new development of large-scale sequencing allows analysis of 400 genes simultaneously with a minimal quantity of DNA of 50 ng DNA. High volume for sequencing is also offered with a mean value of 1.5 μg. That opens a window to large-scale molecular analysis from a single EUS-FNA materials and from a single patient.

RNA extraction
While material collected from pancreatic tumor or inflammatory tissue is less exposed to RNAse digestion as compared to normal pancreatic tissue, the risk of degradation is very high if one wants to analyze high-quality RNA. From a practical point of view, cytological samples should be immediately stored in transport medium (such as RNable) and frozen at -25 ℃ until use. After centrifugation, total RNA can be extracted using Micro kits (for example RNeasy from Qiagen) followed by DNAse treatment. At this crucial stage, RNA quality and quantity should be determined with specific bioanalyzer (for example Biorad Experian analyser and Agilent Technologies). RNA samples that are highly degraded (RNA 18S/28S ratio less than 1) or with a quantity lower than 5 ng should be discarded. Indeed, degraded RNA are not suitable for reverse transcription or amplification. In our experience, near 50% of FNA materials appears non available for a reliable mRNA analysis. For assay of

Table 1 Main studies investigating Kras mutation assay on specimens obtained by endoscopic ultrasound-guided fine-needle aspiration for the differential diagnosis between pancreatic carcinoma and pseudo-tumorous chronic pancreatitis				
Ref.	**Patient**	**Sensitivity (%)**	**Specificity (%)**	**Overall accuracy (%)**
	PC/CP	Cytopathology alone/*Kras* + cytoP	Cytopathology alone/*Kras* + cytoP	Cytopathology alone/*Kras* + cytoP
Tada *et al*[31]	28/8	62/81	100/100	71/85
Pellisé *et al*[35]	33/24	97/97	100/100	84/98
Takahashi *et al*[34]	62/15	84/94	100/100	CytoP alone: 58
Maluf-Filho *et al*[33]	57/11	82/90	97/47	59/89
Bournet *et al*[28]	129/27	83/88	100/100	72/90
Reicher *et al*[30]1	34/16	88	94	90
Ogura *et al*[29]	307/47	87/93	100/100	89/94
Ginestà *et al*[32]	43/18	76/86	100/100	82/90
Bournet *et al*[39]2	104/72	71/90	100/99	84/94

[1]Combination of *Kras* + cytoP + FISH/Fluorescence *in situ* hybridization; [2]PC *vs* others malignant and benign pancreatic lesions. PC: Pancreatic carcinoma; CP: Chronic pancreatitis; CytoP: Cytopathology.

qPCR for 3 to 5 molecular markers an amplification is theoretically not required but if analysis on a larger panel of molecular targets is mandatory, amplification should be performed. Using 5 ng of total RNA (not degraded) is sufficient to perform RNA amplification kits (for instance Full Spectrum Kit) that permit up to 500-fold amplification with satisfactory reproducibility and reliability. In other terms, the RNA amplification from EUS-FNA material preserves the pattern of gene expression and is not influenced by the origin of the sample[23]. We had thus apply the technology of Taqmann Low Density Array to assess simultaneously the quantitative expression of 20 to 50 genes from EUS-FNA cellular materials (see below).

Micro RNA extraction

Interestingly, microRNAs are small molecules (19-25 nucleotides) of non coding RNA with high stability (less prompted to be degraded by RNase) in tissues and fluids. Moreover, they can be quantified in very low amounts of material and in highly degraded samples. Prior to microRNA analysis, tissues can be stored either frozen, or formalin-fixed and paraffin-embedded or in specific medium such as RNARetain[24]. It is important to mention that microRNA analysis of pancreatic FNA samples is possible but still in its infancy and may prove essential to help clinicians for the diagnosis of pancreatic lesions.

GENETIC MARKERS

Kras oncogene

The molecular mechanisms underlying pancreatic oncogenesis remain partially understood. However, several genetic alterations are well characterized in PDAC such as codon-12 *Kras* mutation (75% to 95%) and to a less extend *p16 (CDKN2A, INK4)*, *DPC4* and *p53* gene mutations[25,26] associated to a loss of heterozygosity of respectively 9p21, 18q and 17p. These somatic genetic alterations are also detected in pre-cancerous lesion of PDAC as intraepithelial neoplasias (PanIN) and intraductal papillary mucinous neoplasm (IPMN)[26]. Previous studies conducted by we and others on pure pancreatic

juice obtained by ERCP concluded that *Kras* mutation was found in 60% to 65 % of PDAC[19,20]. Moreover, additional *p16* and *DPC4* mutations analysis in pure pancreatic juice did not improve the sensitivity and specificity of *Kras* mutation analysis alone for diagnosis of PADC and to differentiate PDAC from CP[21]. Several research groups, including ours, have demonstrated that *KRAS* mutations can be detected in cellular materials obtained by EUS-FNA[27-37]. *Kras*-mutation analysis after EUS-FNA appears to be highly accurate at differentiating benign *vs* malignant pancreatic solid lesions[27-35].

We have conducted a multicenter prospective study to assess whether combining EUS-FNA with *KRAS*-mutation analysis could facilitate a differential diagnosis between PDAC and CP in a subgroup of patients with pseudo-tumorous forms[28]. We concluded that, when facing to clinical and radiological presentations of pseudo-tumorous CP, both pathological analyses (inflammation, fibrosis) and wild-type *Kras* are evocative of benignity. Based on the combination of cytopathological (including a second biopsy in case of negative results at the first biopsy) and *Kras* mutation analysis a medical or surgical conservative treatment can be applied. Otherwise, unnecessary pancreatic resection could be avoided. Conversely, when facing a clinical and radiological presentation of CP the presence of mutated *Kras* at EUS-FNA may justify a second biopsy and a follow up to rule out a PADC.

Whether the combination of EUS-FNA plus the *Kras*-mutation assay can improve diagnosis of malignant pancreatic tumors is still debatable. However, several studies have suggested that combining cytopathology and *Kras*-mutation analysis, improves the diagnosis of PDAC and malignancy (Table 1). This appears crucial in case of inconclusive or doubtful diagnosis at cytopathology. Inconclusive specimens were defined as the presence of coagulum with normal cells or acellular samples. Doubtful samples can be defined by the presence of atypia and/or low-grade dysplasia. Even if molecular biology cannot replace histology, the presence of a *Kras* mutation in EUS-FNA material indicates several possibilities: either immediate re-reading of the cytopathology (especially if doubtful) or a rapid indication from a sec-
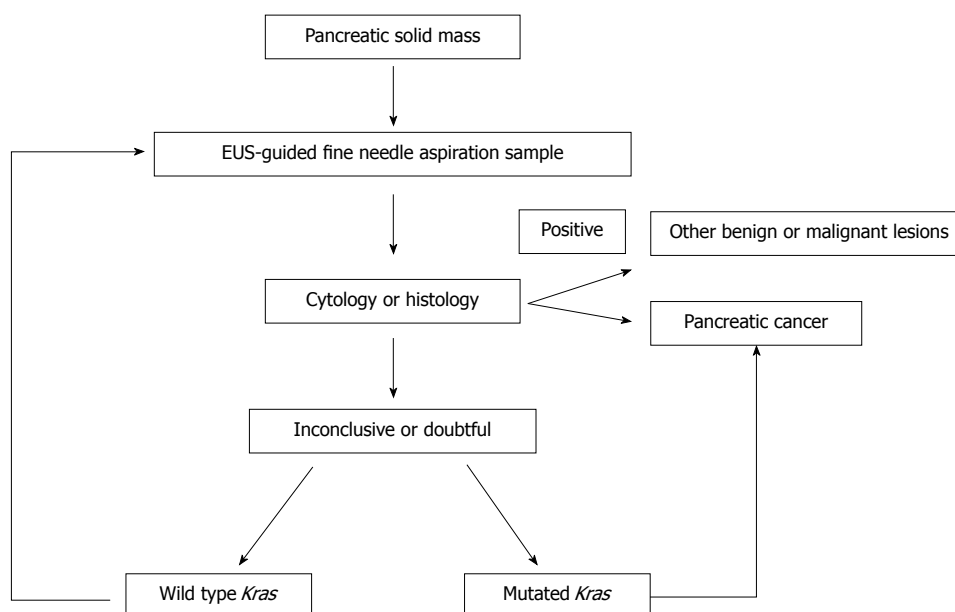
Figure 1 Integration of *Kras* mutation assay coupled to cytopathology in the algorithm of diagnosis of pancreatic cancer using endoscopic ultrasound-guided fine needle aspiration. EUS: Endoscopic ultrasound.

ond FNA, or surgery. In addition, from a clinical point of view, reducing false-negative diagnoses avoids the delay of making decisions, improves patients' treatment, and reduces loss of surgical resectability. Conversely, in cases where there is an inconclusive EUS-FNA specimen, the presence of wild-type *Kras* may be evocative of benignity. Figure 1, integrates these conclusions in a proposed algorithm that include *Kras* mutation assay in the diagnosis approach of pancreatic solid masses using EUS-guided FNA. Because *Kras* analysis is now widely available, due to its use as a predictive marker for anti-EGFR therapy in colon cancer, this diagnostic tool could also be applied to help clinicians manage solid pancreatic masses. *Kras* assay has been improved by means of Taqmann Allelic discrimination that is cheaper, faster and more selective than other previous methods[38]. We have conducted recently a prospective study that included 186 patients with a pancreatic mass (including 104 PDAC, 22 other malignant pancreatic tumors and 60 benign lesions). Cytopathology and *Kras* mutations, using TaqMan® allelic discrimination, were performed on EUS-guided FNA material. We concluded that EUS-guided FNA plus *Kras*-mutation analysis, using allelic discrimination, is accurate and improves the diagnosis of pancreatic adenocarcinoma when pathology is inconclusive or doubtful (Table 1)[39]. In addition, we also confirmed that, when facing a clinical, radiological presentation of pseudo-tumorous chronic pancreatitis (including an evocative cytopathology), identification of wild-type *Kras* can rule out malignant transformation[39]. A retrospective study that included PDAC patients but also patients with an autoimmune pancreatitis reported also that a *Kras* mutation in EUS-guided FNA material from a pancreatic mass is associated with malignancy and may help discriminate from benign conditions such

as autoimmune pancreatitis. In the study from Khalid *et al*[36] all of autoimmune pancreatitis cases had a wild type *Kras*.

Several groups, including ours, investigated whether presence or not of *Kras* mutation can influence prognosis of PDAC, especially in advanced tumors that are only investigated through EUS-FNA. Four recent studies have reported *Kras* mutations in advanced PDAC, though no correlation was found between *Kras* mutations and patient survival[40-43]. Conversely, three other published studies (one that included patients with resected PDAC, and two that included mixed populations with resected and locally advanced/metastatic PDAC) suggest that the presence of *Kras* mutations in tumor tissues have a significant adverse impact on median survival time[44-46]. Therefore, it is still difficult to conclude that the presence of *Kras* mutations influences or does not influence the prognosis of advanced PDAC. To gain further insights and to obtain a definitive conclusion, investigations on a larger cohort of similar patients (to allow strong multivariate analysis) are needed.

Others molecular alterations
Itoi *et al*[47] conducted a p53 immunohistochemical analysis in FNA biopsy specimens obtained from CP and pancreatic cancers. They reported that p53 protein overexpression was observed in 67% of the samples with pancreatic cancer, but not in samples with chronic pancreatitis, and they found that by using the combination of p53 protein overexpression and conventional histological examination, the diagnosis of pancreatic cancers improved as follows: 90% sensitivity, 91% specificity, and 92% accuracy, whereas the conventional histological EUS-FNA diagnostic test statistics for the pancreatic masses were as follows: 76% sensitivity, 91% specific-

ity, and 79% accuracy. Jahng *et al*[48] reported that the combination of p53 and cytology to detect malignancy increased the sensitivity to 51% with 100% specificity, whereas cytology alone had 41% sensitivity and 100% specificity. Salek *et al*[49] reported also that EUS-guided FNA cytology combines with screening of *Kras* mutations and allelic losses of tumor suppressor p16 and DPC4 represents a very sensitive approach particularly in cases where FNA has been inconclusive. Another group recently investigated with the same issue the quantitative analysis of MMR genes[50].

MICRORNA

MicroRNA: from basics to clinics?

MicroRNAs are small non coding RNA that functions as translation inhibitors of messenger RNA mainly following binding to 3'-untranslated region[51-53]. This mechanism is conserved from plants to humans. These molecules are tightly involved in the regulation of many physiological processes. Indeed they regulate more than 30% of mammalian gene products. In addition, microRNAs play important roles in many diseases, including cancer, cardiovascular disease, and immune disorders. Besides their high stability in tissues and fluids, microRNAs can repress the expression of dozens or hundreds of genes, making them an attractive therapeutic target.

MicroRNA expression is finely regulated by epigenetic modification (DNA methylation of promoters encoding for microRNA), change in DNA copy numbers, and genetic mutations[54]. For example miR-21 production is increased by *Kras* (G12D) or EGFR and decreased by TGF-b[55]. For epigenetic regulation Choi *et al*[56] described in 2012 the DNA methylation of promoter encoding for many microRNAs as a physiological process for mesenchymal stem cell differentiation. As described previously, microRNAs are very stable in tissues and fluids (urine, plasma, saliva). This is a key advantage as compare to protein or mRNA. That is why microRNAs are an emerging class of biomarkers in physiological and pathological processes, including pancreatic diseases.

MicroRNA and pancreatic cancer

microRNA expression is profoundly altered in cancer or is strongly modulated during carcinogenesis. MicroRNAs can be organized in two classes; the oncomicroRNAs which are upregulated in cancer (miR-155, miR-21)[57] and the tumor suppressor microRNAs (let-7 family) which are down regulated in cancer cells[58].

Concerning pancreatic cancer, many articles described that there is an early aberrant microRNA production in pancreatic carcinogenesis and more precisely in the development of precancerous lesions called PanIN. Indeed, the production of miR-21, miR-221, miR-222, and let-7a increased with human PanIN grade, with peak production occurring in hyperplastic PanIN-2/3 lesions[55]. Epigenetic regulation of microRNA is also described to modulate microRNA expression during pancreatic

carcinogenesis. For example, miR-148 is down regulated due to an hypermethylation of its DNA[59]. These early disturbances in the expression of microRNA persist then partly in advanced pancreatic cancer stages. In addition, many recent reports describe the alteration of microRNA expression in IPMNs, well-described non-invasive precursor lesions of pancreatic cancer[60]. Such approach may aid in diagnosis and surgical treatment decisions for patients with pancreatic cystic lesions. Taken together, microRNAs could be the ultimate biomarkers at the clinical level for the early diagnosis of pancreatic cancer and would thus allow tumor resection that is usually associated with the best prognosis.

Wang *et al*[61] were the first to report the detection of microRNA in the blood of pancreatic cancer patients. Interestingly, microRNA profiling in plasma can differentiate pancreatic cancer patients from healthy controls. They demonstrate that the plasma levels of panel of four microRNAs (miR-21, miR-210, miR-196a and miR-155) reveal a sensitivity of 64% and a specificity of 89% for pancreatic cancer. In addition, expression profiles of microRNAs may also be very informative not only to discriminate pancreatic cancer from the normal pancreas, but also for the differential diagnosis of chronic pancreatitis. This shows the interest of microRNAs as diagnostic tool in biological fluids in a non-invasive manner.

MicroRNA have been described as key players in pancreatic cancer development but above in pancreatic cancer cell chemoresistance. The mechanisms involved in drug resistance of cancer cell include alteration of drug target, altered regulation of the cell cycle and apoptosis, increased DNA damage repair and ejection of the drug from the cell by drug efflux pumps. Interestingly, microRNAs can influence the drug response by regulating all of these cellular processes[62]. MiR-21, miR-146, miR205, miR10b, miR-7 and many others microRNAs are implicated in these phenomenon. In this context, microRNAs can serve not only as a valuable therapeutic target but also as a predictive marker for a large number of diseases including pancreatic cancer. The study of microRNA expression in tumors may also lead to the identification of different molecular subtypes of pancreatic cancer that may provide insight into selection of patients likely to benefit from therapies. Nevertheless, whether this will translate into clinical applications is still highly debated.

Some microRNAs are not only predictive and diagnostic markers but also prognostic markers. Indeed, Bloomston *et al*[63] originally reported that 6 microRNAs (miR-452, miR-105, miR-127, miR-518a-2, miR-187, miR-30a-3p) are over-expressed in the patients with a longer survival (greater than 2 years). In addition, Yu *et al*[64] reported that patients with high levels of miR-200c expression present with significantly better survival rates than those with low levels of miR-200c expression.

To conclude microRNA expression signature may be informative for the diagnosis, prognosis and predictive of pancreatic cancer[65]. In other words microRNAs

Table 2 Main studies with expression of miRNA in endoscopic ultrasound-guided fine needle aspiration specimens

Ref.	miRNA	FNA	Possible clinical implication
Torrisani *et al*[58]	Let-7a	X ↓	Diagnosis
Hanoun *et al*[22]	miR-148b	X ↓	Diagnosis
Szafranska *et al*[24]			
Szafranska *et al*[24]	miR-196a	X ↑	Diagnosis
Szafranska-Schwarzbach *et al*[66]			
Szafranska *et al*[24]	miR-217	X ↓	Diagnosis
Szafranska-Schwarzbach *et al*[66]			
Du Rieu *et al*[55]	miR-21	X ↑	Diagnosis, prognosis, response to treatment
Bloomston *et al*[63]			
Preis *et al*[67]	miR-10b	X ↑	Prognosis, response to treatment

X ↑: Up regulated; X ↓: Down regulated. FNA: Fine needle aspiration.

appear to be reliable biomarkers to assist clinicians in all stages of care for patients with pancreatic cancer. Thus, microRNAs are expected in the future to prove specific and/or sensitive as a long-awaited screening tool for pancreatic cancer.

MicroRNA detection EUS-FNA

Nowadays, the vast majority of pancreatic cancer patients have metastatic and/or locally advanced diseases at the time of diagnosis; in other words, these patients are not eligible for curative resection which explains the limited access to pancreatic tissue specimens. As explain before, endoscopic ultrasound-guided fine needle aspiration-biopsy is the most widely used approach for cytological and histological material sampling in this situation. Szafranska *et al*[24] revealed that microRNA and more precisely, miR-196a and miR-217 expression analyses from FNA material can discriminate pancreatic cancer from benign lesion with a high sensitivity (90%) and specificity (100%). These results paved the way to the first development of a molecular test using microRNA for the differential diagnosis of pancreatic cancer[66].

Preis's[67] group has demonstrated that miR-10b and miR-21, two well-characterized onko microRNAs, are over expressed in the FNA material from pancreatic cancer patients. MiR-10b is now classified as a novel and powerful diagnostic biomarker for pancreatic cancer. In addition, reduced expression of miR-10b is associated with improved response to multimodality neoadjuvant therapy, surgical resection, time to metastasis, and increased survival. Thus, miR-10b may serve as a novel diagnostic and prognostic biomarker in PDAC and as a tool for predicting response to therapy.

Then, we recently demonstrated that let-7a tumor suppressor microRNA expression is repressed[58] in FNA material of pancreatic cancer and that the measurement of hypermethylation of microRNA miT-148a encoding DNA region is potentially useful to differentiate pancreatic cancer and pseudo-tumor forms of chronic pancreatitis[22].

To conclude, microRNAs are very promising emerging molecular markers in pancreatic cancer that can be quantified in EUS-FNA specimens. Table 2 resumes clinical applications of microRNAs in FNA material. However, forthcoming prospective studies are needed to ask whether microRNAs may translate into reliable biomarkers for pancreatic cancer management.

MUCINS AND MARKERS OF INVASION

Expression of mucins

Mucine (MUC) are the main components of mucus. They are synthesized and secreted by specialized cells of the epithelium and in some case, by non mucin-secreting cells. MUC are membrane-tethered high molecular weight glycoprotein, and frequently overexpressed in PDAC. Mucins have been implicated in tumorigenicity, invasiveness, metastasis and drug resistance through their characteristic *O*-linked and *N*-linked oligosaccharides, extended structures and unique domains. MUC are classified into three categories, membrane associated mucins with MUC1, MUC3 or MUC4, gel-forming mucins with MUC 2 or MUC5AC and soluble mucin with MUC7. MUC are expressed in normal pancreatic tissue, PDAC or precusors lesions as IPMN or PanIN[68]. The MUC expression profile has a hight value for the diagnosis of PDAC (especially MUC1) and several studies implicated these markers in the prognosis and outcome of patient. From samples obtained under EUS FNA, MUC can be detected using immunohistochemistry[69,70]. By this way, Nagata *et al*[68] investigated the expression of MUC in various pancreatic tissues. MUC1 and MUC6 are expressed in normal pancreatic tissues while MUC 2 and MUC 5AC are never expressed. The expression profile of MUC in IPMN is different between the different subtypes of IPMN. IPMN of intestinal type display a high expression of MUC2 and MUC5 AC while IPMN of pancreaticobiliary type has a low expression of MUC2 and a high expression of MUC 5AC. In PDAC tissues, MUC1 has a high expression but no expression of MUC2. Wang *et al*[71], after immunohistochemistry on EUS-FNA samples demonstrated the expression of MUC1, MUC2 and MUC5AC in PDAC and in benign pancreatic disease samples. They investigated the diagnosis value of cytology analysis alone *vs* combination of cytology together with the presence of MUC1 or MUC 5AC. They concluded that the combination of cytology and MUC1+ or MUC5AC+ provide higher sensibility and accuracy for the diagnosis of PDAC (Table 3).

Expression of factors implicated in tumor invasion

The identification of others biomarkers has been proposed from samples of pancreatic tissue obtained by EUS-FNA. Indeed the quality and the amount of cellular pancreatic samples obtained by EUS-FNA allow immunohistochemistry thanks to cellblocks but also the extraction of RNA to perform Real Time Reverse Transcription Polymerase Chain Reaction.

We previously performed an expression study using cDNA macro array of pancreatic cancer cells and PDAC

Table 3	Main studies investigated expression of Mucin and molecular markers for the diagnosis of pancreatic cancer using endoscopic ultrasound-guided fine needle aspiration materials				
Molecular markers	**Methods for analysis**	**Sensitivity (%)**	**Specificity (%)**	**Accuracy (%)**	**Ref.**
MUC1+/MUC2-/MUC5AC+	IHC	70	100	75	70
CytoP + MUC5AC	IHC	90	93	91	71
CytoP + MUC1		85	100	89	
MSLN, UPAR	qRT-PCR	100	94	-	77
S100P	IHC	90	90	87	78
MSLN		62	74	66	
S100P + KRT7	qRT-PCR	80	77	-	23
cytoP alone	qRT-PCR	68/88	100/90	75/89	73
cytoP alone/cytoP + S100A6					

CytoP: Cytopathology; IHC: Immunohistochemistry; qRT PCR: Quantitative reverse transcription polymerase chain reaction; MUC: Mucin; MSLN: Mesothelin; UPAR: Urokinase plasminogen activator receptor.

tissues. Following this DNA chip study, RT-QPCR validated the increased expression of LCN2 (lipocalin 2) and for the first time PLAT (tissue-type plasminogen activator or tPA) in PDAC as compared with normal pancreas. Following, PLAT and LCN2 transcripts obtained through EUS-guided FNA from patients with PDAC showed a significant increased expression levels in comparison with those found in normal tissues, indicating that a sufficient amount of high quality RNA can be obtained with this technique[72].

Subsequently we conducted a prospective multicenter study using dedicated TaqMan Low Density Array technology on EUS-FNA materials[23]. We quantified candidate gene expression in biopsies sampled from 44 locally advanced and/or metastatic pancreatic carcinoma and from 17 pseudotumoural chronic pancreatitis. We found that eight genes (*S100P, PLAT/Plasminogen Activator Tissue, PLAU/PLasmine Activator Urokinase, MSLN/Mesothelin, Matrix MetalloProteins 7* and *11, KRT7* and *17/Keratin*) were significantly over expressed in pancreatic cancer samples when compared to pseudotumoural chronic pancreatitis. The area under receiver operating curve establishes the clinical validity of the potential diagnostic markers identified in this study (values ranging from 0.69 to 0.76). In addition, combination of S100P (calcium binding protein P) and KRT7 gave better diagnosis performances (Table 3). We demonstrate that molecular studies on EUS-guided FNA material are feasible for the identification and quantification of markers in PDAC patients diagnosed with non-resectable tumours. Using low-density array, we isolated a molecular signature of advanced pancreatic carcinoma including mostly cancer invasion-related genes[23]. Zihao *et al*[73] demonstrated the interest of S100A6 for the diagnosis of pancreatic cancer. The material used RNA extracted for quantification of gene expression by RT PCR and S100A6 immunohistochemistry to validate the expression. Deng *et al*[74] demonstrated also a nuclear or cytoplasmic staining for S100P and it was specific for pancreatic cancer with 100% diagnostic sensibility. Kosarac *et al*[75] obtained similar results. These different biomarkers as calcium binding proteins such as S100P (that are associated to metastase and poor prognosis) may contributed in positive diagnosis of

pancreatic cancer but also in differential diagnostic with benign pancreatic disease[76-78].

MARKERS FOR THE TREATMENT EFFICACY

Gemcitabine is transported into cells predominantly by human equilibrative nucleoside transporters[79]. A deficiency of activity of one of them, hENT1, conferred high-level resistance to the toxicity of gemcitabine[80-83], and patients with PDAC that have detectable hENT1 or high *hENT1* gene expression have significantly prolonged survival after gemcitabine chemotherapy when given as adjuvant treatment after resection[84,85]. After transport, Gemcitabine must be sequentially converted into mono- di- or triphosphorylated forms to exert its full cytotoxic activity. In this cascade, the first two steps of phosphorylation are rate-limiting. In mammalian cells, gemcitabine conversion into gemcitabine monophosphate is performed by the deoxycytidine kinase (DCK)[80-81]. Then, the Uridylate monophosphate kinase yields gemcitabine diphosphate[81]. Gemcitabine derivatives exhibit antitumor activity either by interfering with intracellular nucleotide pools, or through direct inhibition of DNA synthesis. Resistance to gemcitabine has been reported to involve a deficiency in DCK enzyme, a decrease in nucleoside transport and an overexpression of ribonucleotide reductase. After cellular entry, gemcitabine must be phosphorylated by deoxycytidine kinase (dCK), which is a rate limiting step. We previously demonstrated that down-regulation of dCK specifically enhanced acquired resistance to gemcitabine in pancreatic cancer cells, whereas transfection of wild-type dCK restored sensitivity to the drug[83]. Conversely, active metabolites of gemcitabine are reduced by 5'-nucleotidase, and gemcitabine itself is inactivated by cytidine deaminase (CDA). High levels of these catabolic enzymes are associated with resistance to the drug. Ribonucleic Reductase (RR) is a dimeric enzyme composed of regulatory subunit M1 and catalytic subunit M2. PDAC patients with high levels of RRM1 expression had poor survival rates after gemcitabine treatment. Moreover, RRM2 gene silencing

by RNA interference is an effective therapeutic adjunct to gemcitabine treatment. These data suggest that the genes encoding proteins involved in the transport and metabolism of gemcitabine and in the metabolism of targets can be potential candidates to predict sensitivity to gemcitabine.

Fujita *et al*[84] investigated 70 patients with PDAC. Of the 70 patients, 40 received gemcitabine-based adjuvant chemotherapy. They measured hENT1, dCK, CDA, RRM1, and RRM2 mRNA levels by quantitative real-time reverse transcription-polymerase chain reaction in endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) cytological specimens. High dCK, low RRM2 groups had a significantly longer disease-free survival in the gemcitabine-treated group[85]. Itoi *et al*[86] assessed 35 PDAC biopsy specimens for RRM2 expression levels. In the low RRM2 expression group, a complete response was observed in one patient, and a partial response was observed in eight patients. In contrast, in the high RRM2 expression group, complete response was not observed. In the work from Ashida *et al*[87] mRNAs were extracted from 35 unresectable pancreatic adenocarcinoma tissues obtained by EUS-FNA before GEM-treatment. Among these GEM sensitivity-related genes, dCK alone showed a significant correlation with Gemcitabine efficacy. Among all molecules that are crucial for Gemcitabine intracellular transport and metabolism, hENT1, dCK and RRM2 appear important. If their expression has been studied at the mRNA levels on EUS-FNA, immunohistochemistry on these materials remains to be validated.

CONCLUSION

Both clinician and scientist take benefit from cellular and tissue material sampled under EUS-FNA in PDAC patients. The progress of molecular biology authorizes now extraction of DNA, mRNA and miRNA. After amplification identification of genetic abnormalities and quantification of biomarkers for improvement of diagnosis, prognosis and hopefully treatment together with a better knowledge of pancreatic carcinogenesis especially in locally advanced pancreatic adenocarcinoma. Among these markers, *Kras* oncogene assay appears now the most robust for improvement of positive and differential diagnosis of pancreatic cancer especially when FNA are inconclusive or doubtful. Clinical implication or miRNA, Mucins and markers of invasion is still debated. Future molecular developments may open windows towards personalized treatments after molecular characterization of a single patient.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Translational research in pancreatic ductal adenocarcinoma: Current evidence and future concepts

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Abstract

Pancreatic ductal adenocarcinoma (PDA) is one of the major causes for cancer death worldwide. Treatment of metastatic disease remains challenging as only certain patients benefit from advances made with the intensified chemotherapy regimen folinic acid, irinotecan and oxaliplatin, the epidermal growth factor receptor inhibitor erlotinib or the recently FDA-approved nab-paclitaxel. Up to date, no established approach for prediction of treatment response or specific treatment allocation exists. Translational research was able to identify a number of potential biomarkers that might help to improve the dismal prognosis of PDA by facilitating upfront treatment allocation. This topic highlight is focused on current evidence on potential biomarkers for tumor biology, prognosis and prediction of treatment efficacy.

Core tip: Recent advances in the treatment of pancreatic ductal adenocarcinoma (PDA) have been made using the intensified chemotherapy regimen folinic acid, irinotecan and oxaliplatin, the recently FDA-approved nab-paclitaxel and the epidermal growth factor receptor-inhibitor erlotinib. Yet overall prognosis of PDA remains poor. To further improve outcome of PDA, innovative strategies are needed to identify patient subgroups that benefit most from specific regimens. This topic highlight focuses on potential biomarkers to identify patients that benefit from treatment with erlotinib (*e.g.* KRAS, AKT, ERK, p53), gemcitabine (hENT1, RRM1, dCK), nab-paclitaxel (SPARC) or angiogenesis inhibitors. Additional biomarkers of tumor biology (like SMAD4 and CXCR4) and future concepts of translational research in PDA are also discussed.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) constitutes

the fifth leading cause of cancer death accounting for approximately 227000 annual deaths worldwide[1-4]. It is only curable by surgical resection which is feasible in about 15%-20% of all patients[4]. Non-resectable patients usually receive palliative chemotherapy with gemcitabine-based combinations[5]. However, these combinations often fail to offer long-term disease control resulting in a poor five-year survival rate of about 4%[4]. Advances in specific patient populations have been made using the tyrosine kinase inhibitor erlotinib and an intensive treatment regimen consisting of 5-fluorouracil (5-FU), folinic acid, irinotecan and oxaliplatin (FOLFIRINOX). While effects on the overall population are minimal for erlotinib, intensive chemotherapy with FOLFIRINOX is tolerated by certain patients only[6,7]. Treatment options proven to be beneficial in other cancer entities like the vascular endothelial growth factor (VEGF) inhibitor bevacizumab have failed to improve survival in an unselected PDA population[8]. Preclinical data propose that innovative agents like the recently FDA-approved albumin bound nab-paclitaxel might be dependent on expression of specific proteins, suggesting predefined patient subgroups as major beneficiaries[9]. Hence new biomarkers are urgently needed for treatment allocation and identification of patient subgroups that might benefit from alternative treatment strategies[10]. This topic highlight summarizes and assesses current evidence from translational studies on biomarkers for tumor biology, prognosis and prediction of treatment response in PDA.

Biomarkers for tumor biology and prognosis

SMAD4: SMAD4/DPC4 is a protein involved in intracellular transforming growth factor-b1 signaling[11]. In PDA differing functions as biomarker have been ascribed to SMAD4. Based on findings in rapid autopsies performed on patients previously diagnosed with stage I to IV PDA, Iacobuzio-Donahue *et al*[12] suggested SMAD4 as a biomarker for metastatic pattern of PDA. They determined presence or absence of intact SMAD4 using immunochemistry in PDA of 65 patients. Abnormal immunostaining of SMAD4 was found in 41 patients (63%). Absence of intact SMAD4 was significantly more frequent in metastatic disease (78%) and significantly reduced in locally destructive disease (22%) (*P* = 0.007). Oshima *et al*[13] screened 106 patients with PDA who had undergone surgical resection for mutations in different genes including the *SMAD4* gene. Abnormal immunolabeling for SMAD4 was detected in 64 patients (60%). Using univariate analysis, a significant correlation between tumor size (*P* = 0.006), lymphatic invasion (*P* = 0.033), lymph node metastasis (*P* = 0.006) and abnormal immunostaining for SMAD4 was found. Overall survival for patients with intact *vs* mutant SMAD4 was 30.1 mo *vs* 18.3 mo respectively (*P* < 0.001). Within a multivariate analysis mutant SMAD4 was found to be a significant and independent poor prognostic factor for both disease free and overall survival. In a different study Bachet and co-workers examined tumor samples of 471 patients with resected PDA and assessed SMAD4 status by tissue microarray analyses. Patients with mutant

SMAD4 significantly benefited from adjuvant chemotherapy (hazard ratio for death compared to untreated patients = 0.59; 95%CI: 0.42-0.82; *P* = 0.002), whereas no significant beneficial effect of adjuvant treatment was witnessed for SMAD4 wild-type status (hazard ratio for death = 0.85; 95%CI: 0.49-1.46; *P* = 0.552). While disputing a correlation between metastatic pattern and SMAD4 status, the authors conclude that SMAD4 might also predict adjuvant treatment response[14]. Using multivariate analysis Winter *et al*[15] recently examined the correlation between SMAD4 status and different clinical criteria in 127 patients with resected PDA. In harsh contrast to earlier findings they found neither a correlation between SMAD4 and metastatic pattern nor a correlation between SMAD4 and overall survival (Table 1).

CXCR4: Chemokines are small cytokines capable of inducing chemotaxis. They exert their effects *via* specific chemokine receptors found on various cell types including immune and tumor cells. The chemokine receptor CXCR4 has been described to be widely expressed in different cancer types[16]. *Via* interaction with its ligand, the chemokine CXCL12 (SDF-1α) is believed to promote tumor growth, angiogenesis and tumor dissemination[17]. In PDA two retrospective studies concluded CXCR4 to be a significant and independent poor prognostic factor for overall survival while a third study found no significant correlation between overall survival and CXCR4[14,18,19]. The CXCR4 ligand CXCL12 has recently been reported to be a predictive marker for treatment response to bevacizumab as discussed below[20]. Additional research is clearly necessary to identify the potential of CXCR4 and its ligand in predicting treatment response and prognosis in PDA.

Predictive biomarkers of the epidermal growth factor receptor pathway

The oral epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor erlotinib modestly improves survival in an unselected patient population with metastatic PDA. However, a significant survival benefit from erlotinib treatment is observed for patients developing skin rash[5]. Erlotinib exerts its effects by inhibiting intracellular receptor transphosphorylation of the ErbB1/HER1 receptor[21]. Translational studies therefore aimed to identify EGFR polymorphisms and gene amplifications predictive for erlotinib treatment. Despite of promising findings in pre-clinical and early clinical studies, translational subgroup analyses from prospective clinical trials failed to reveal a significant correlation between genetic EGFR alterations or overexpression and treatment response to erlotinib up to now[22]. Recent investigational approaches on identifying predictive EGFR pathway biomarkers have therefore focused on downstream EGFR signaling such as the PI3K-AKT-PTEN network or the RAS-RAF-MAPK-MEK-ERK cascade.

KRAS: Mutations in members of the *RAS* gene family such as v-Ki-ras2 Kirsten rat sarcoma viral oncogene ho-

Table 1 Summary of current evidence on selected biomarkers in pancreatic ductal adenocarcinoma discussed in this topic highlight

Ref.	Biomarker	Prognostic	Predictive (for)	Dissemination pattern
Iacobuzio-Donahue *et al*[12]	SMAD4	N/A	N/A	+
Oshima *et al*[13]		+	N/A	+
Bachet *et al*[14]		-	+ (adjuvant chemotherapy)	-
Winter *et al*[15]		-	N/A	-
Bachet *et al*[14]	CXCR4	+	-	+
Maréchal *et al*[18]		+	N/A	+
Gebauer *et al*[19]		-	N/A	-
Lee *et al*[27]	KRAS	+	N/A	N/A
Shin *et al*[28]		+	N/A	N/A
Ogura *et al*[29]		+	N/A	N/A
Boeck *et al*[22,31]		+	? (erlotinib)	N/A
Kim *et al*[24]		-	+ (erlotinib)	N/A
da Cunha Santos *et al*[32]		-	-	N/A
Oliveira-Cunha *et al*[33]		-	N/A	N/A
Farrell *et al*[48]	hENT1	-	+ (adjuvant gemcitabine)	N/A
Morinaga *et al*[49]		-	+ (adjuvant gemcitabine)	N/A
Maréchal *et al*[50]		-	+ (adjuvant gemcitabine)	N/A
Greenhalf *et al*[52]		-	+ (adjuvant gemcitabine)	N/A
Jordheim *et al*[51]		-	+ (review on 18 studies on adjuvant gemcitabine)	N/A
Poplin *et al*[53]		-	- (gemcitabine in metastatic PDA)	N/A

(+): Results suggest that the respective molecule might serve as a biomarker; (-): No evidence found that the respective molecule might serve as a biomarker; (?): Unclear whether the respective molecule could serve as a biomarker. N/A: No data available; in cited study; PDA: Pancreatic ductal adenocarcinoma.

molog (KRAS) are frequently observed in different types of human cancers[23]. The highest frequency of mutant KRAS can be found in PDA. It has been reported that mutant KRAS is present in up to 90% of all PDA[10,24]. Single point mutations in codon 12, 13, 59 or 61 of exon 2 and exon 3 of the KRAS oncogene impair intrinsic GTPase activity of KRAS and lead to a permanent active KRAS signaling pathway, resulting in sustained proliferation and survival of cells[23]. KRAS has been described as a predictive biomarker for treatment success by inhibitors of the EGFR pathway such as the small-molecule drug erlotinib or the monoclonal antibodies cetuximab and panitumumab in metastatic non-small cell lung and colorectal cancer[25,26]. So far, the value of KRAS as biomarker in PDA has not been clearly established. In a retrospective study performed by Lee *et al*[27], KRAS status of 66 patients with metastatic ($n = 61$) or locally advanced ($n = 5$) PDA was analyzed. The majority of patients ($n = 64$) had received first-line chemotherapy with gemcitabine alone or gemcitabine in combination with capecitabine, uracil/tegafur (UFT) or cisplatin. In a total of 42 patients (64%) KRAS mutations were found (codon 12: $n = 41$, codon 63: $n = 1$). Comparison between patients with a mutation in codon 12 of the KRAS oncogene and wild-type KRAS showed a significant reduced overall survival for patients with mutant KRAS (9.1 mo *vs* 13.4 mo, $P = 0.03$). In conclusion Lee *et al*[27] suggested that KRAS might be of value as a prognostic biomarker in PDA. In the largest retrospective study conducted on KRAS as a biomarker in PDA so far, Shin *et al*[28] analyzed KRAS status of 234 resected PDA patients by polymerase chain reaction. Mutant KRAS was present in 126 patients (55%). Using multivariate analysis mutant KRAS was found to be significantly correlated to poor prognosis. In a different study by Ogura *et al*[29] similar findings were reported. Of

note neither Shin *et al*[28] nor Ogura *et al*[29] commented on applied chemotherapy regimens in the study population, making it impossible to distinct between a predictive and merely prognostic correlation. The AIO-PK0104 study was a large, multicenter phase III trial in advanced PDA conducted by the German AIO study group[30]. In a post-hoc analysis of AIO-PK0104 the wild-type KRAS status was found to be significantly correlated with an improved overall survival (hazard ratio for death for wild-type compared to mutant KRAS = 1.68; $P = 0.005$). Owing to the study design (all patients received erlotinib plus either capecitabine or gemcitabine as first-line chemotherapy) it was impossible to directly distinguish between a prognostic and predictive correlation[22]. However, within an exploratory analysis no significant correlation of KRAS status with objective response to erlotinib-containing first-line therapy was found, indicating that KRAS may not serve as a predictive but rather as a prognostic biomarker for overall survival in PDA[31]. Contrary to these observations, Kim *et al*[24] screened tumor samples of 136 patients with metastatic ($n = 112$) or locally advanced ($n = 24$) PDA, who had received first-line therapy with gemcitabine alone ($n = 22$) or a combination of gemcitabine with either erlotinib ($n = 70$), capecitabine ($n = 31$) or UFT ($n = 13$). In 71 patients (52%) mutations in codon 12 ($n = 70$) or codon 61 ($n = 1$) of the KRAS oncogene were found. Post-hoc analysis showed a significant difference in overall survival between patients with wild-type and mutant KRAS status treated with erlotinib and gemcitabine (9.7 mo *vs* 5.2 mo, $P = 0.002$) whereas no difference in survival was observed in patients treated with regimens without erlotinib (7.0 mo *vs* 7.0 mo, $P = 0.121$). The authors from this Asian study therefore concluded that KRAS might be a predictive but not a prognostic biomarker. In clear contrast to this conclusion

are the findings of da Cunha Santos *et al*[32], who analyzed tumor samples of 117 patients from the erlotinib pivotal trial PA.3. Mutant KRAS was present in 92 patients (79%). Comparison of overall survival showed a non-significant survival benefit for wild-type KRAS patients treated with erlotinib plus gemcitabine *vs* patients treated with gemcitabine plus placebo (6.1 mo *vs* 4.5 mo, $P = 0.34$) while patients in the wild-type KRAS arm showed a trend towards reduced overall survival under anti-EGFR therapy (6.0 mo *vs* 7.4 mo, $P = 0.78$). In a study conducted by Oliveira-Cunha and co-workers the correlation between KRAS status and overall survival in 100 patients with resected pancreatic and periampullary cancer was analyzed. The investigators reported a non-significant shorter overall survival for mutant KRAS patients (22.8 mo *vs* 28.1 mo, $P = 0.88$) and concluded that there is no correlation between KRAS and overall survival[33]. Noteworthy limitations of mentioned studies are retrospective design, lack of data on systemic therapy and vague definition of the cancer subtype investigated (*e.g.* periampullary cancer *vs* PDA). Additional research using prospective biomarker studies with clearly defined patient populations is crucial to clarify the possible use of KRAS as a prognostic or (even more important) as a predictive biomarker for treatment response to erlotinib.

ERK: EGFR signaling through KRAS is dependent on a complex interplay of intracellular proteins like the extracellular signal-regulated-/mitogen-activated protein kinase (ERK/MAPK). Because of its location downstream the RAS-RAF-MEK cascade, ERK might be useful in predicting success of anti-EGFR treatment[34]. Additionally some previous studies in PDA suggested that high ERK expression might be a poor prognostic factor while other studies found no correlation between ERK expression and survival[35-37]. The AIO-PK0104 investigators also examined the correlation between ERK expression and overall survival. Archival tumor tissue samples of 153 patients with advanced PDA who had received an erlotinib-based 1st-line regimen were analyzed using a grading system of cytoplasmic and nuclear phospho(p-)ERK expression ranging from 0 (no expression) to 12 (high expression). A significant increase in the hazard ratio for death by a factor of 1.06 for each pERK score level (0 to 12) was observed (HR = 1.06; 95%CI: 1.0-1.12; $P = 0.05$). As for KRAS (see above) it was not possible to definitely determine whether this correlation is solely prognostic or predictive for erlotinib efficacy due to the design of the trial[38].

AKT: Besides activation of KRAS, dimerization of EGFR activates phosphoinositol-3-kinase (PI3K), resulting in activation of the serine/threonine-specific protein kinase AKT. The active form of AKT, phosphorylated AKT (pAKT) is an important mediator of cell survival and protein synthesis[34]. Its activity is negatively regulated by the tumor suppressor protein phosphatase and tensin homolog (PTEN). A deregulated AKT/PTEN pathway leads to

resistance of cancer cell lines against anti-EGFR treatment *in vitro*[39]. Additionally, a correlation between expression of AKT and overall survival has been described in previous small PDA studies[35,36]. Within AIO-PK0104 tumor samples of 35 patients were categorized based on their pAKT expression level: no difference in progression free or overall survival between PDA patients expressing low or high levels of pAKT was observed[38].

p53: Mutations in the tumor suppressor gene TP53 are an important step in the oncogenesis of most human cancer types. Including PDA, approximately 80% of all malignant tumors embody mutated TP53. Its transcriptional product p53 has been described to interact with the EGFR/KRAS signaling pathway in PDA[34,40,41]. Additionally, p53 was recently also found to potentially serve as an independent predictive biomarker for treatment success with the monoclonal anti-EGFR antibody cetuximab in locally advanced rectal cancer[42]. Pre-liminary findings from 50 patients treated within AIO-PK0104 showed that overall survival was independent of p53 expression; however, progression free survival was significantly reduced in patients with p53 loss (1.8 mo) or overexpression of p53 usually resulting in dominant negative p53 (2.5 mo) compared to normal levels of p53 expression (6 mo)[38]. These pre-liminary findings may provide further evidence that not only a loss of p53 but also its overexpression is an important step in carcinogenesis and might be correlated to poor prognosis as also suggested by other studies[43]. Further research in PDA is necessary to confirm these findings and to clarify whether the observed correlation is of prognostic or predictive nature.

Predictive biomarkers of the VEGF pathway

Angiogenesis inhibitors like the VEGF inhibitor bevacizumab have proven to be beneficial as add-on treatment in multiple cancer entities like colorectal and non-small cell lung cancer[44]. In PDA *antiangiogenic* treatment has failed to show a significant effect in unselected patient populations so far[8]. Lambrechts *et al*[44] identified a possible predictive biomarker to select patients who might benefit from anti-VEGF treatment with bevacizumab: using blood samples collected within the AViTA trial, they genotyped a set of 157 single nucleotide polymorphisms (SNP) in patients who had received gemcitabine and erlotinib plus either bevacizumab (*n* = 77 patients) or placebo (*n* = 77 patients). They identified the rs9582036 SNP in the VEGF receptor 1 region, which significantly correlated with progression-free and overall survival in the bevacizumab-treated group but not in the placebo group. Bevacizumab-treated AA carriers of the rs9582036 SNP showed a median overall survival of 10.2 mo (95%CI: 7.8-14.9) while AC and CC carriers showed a median overall survival of 5.9 mo (95%CI: 4.0-11.5) and 4.7 mo (95%CI: 4.3-NA), respectively. Using a novel multiplex ELISA system Nixon *et al*[20] recently analyzed 31 different factors in plasma samples of 328 patients with metastasized or locally advanced PDA who had re-

ceived gemcitabine plus either bevacizumab or placebo within the CALGB 80303 study; after multivariate analysis three factors were identified as possible predictive biomarkers: while low levels of VEGF-D were found to be predictive for improved outcome in the bevacizumab group, below median levels of CXCL12 (SDF-1alpha) and angiopoetin 2 (Ang2) predicted a lack of benefit in the bevacizumab group. Further prospective biomarker studies are clearly necessary to confirm these pre-liminary findings and to assess the possible benefit of add-on treatment with bevacizumab in a pre-selected PDA population.

Biomarkers for the efficacy of gemcitabine

hENT1: Gemcitabine has been established as standard agent in the adjuvant and palliative chemotherapy setting of PDA more than a decade ago[45]. Gemcitabine uptake by PDA cancer cells is thought to be dependent on human equilibrative nucleoside transporter 1 (hENT1), suggesting hENT1 as a possible predictive biomarker for treatment response to gemcitabine[46,47]. In PDA patients receiving adjuvant treatment with gemcitabine or 5-FU within the RTOG 97-04 study, Farrell *et al*[48] indeed demonstrated that patients treated with gemcitabine showed significant better overall survival if hENT1 was expressed in cancer cells as determined by immunohistochemistry in resected tumors (hazard ratio for death for hENT1 expression *vs* no hENT1 expression: 0.40; 95%CI: 0.22-0.75; $P = 0.03$). No correlation between overall survival and hENT1 expression was found in the 5-FU treated group indicating that hENT1 is a predictive biomarker for treatment response to gemcitabine. In line with these recent findings, Morinaga *et al*[49] previously reported superior overall survival for patients with high levels of hENT1 (22.2 mo) *vs* patients with low hENT1 levels (11.8 mo) in a population that had received adjuvant gemcitabine chemotherapy. In the largest retrospective study to date, Maréchal *et al*[50] collected tumor samples from 434 surgically resected PDA patients among whom 243 had received gemcitabine-based regimens. They found that high hENT1 expression was a strong predictive factor for superior overall survival in the gemcitabine treated group (hazard ratio for death high *vs* low hENT1 expression = 0.43; 95%CI: 0.29-0.63; $P < 0.0001$). In a recent review on 18 (mainly retrospective) clinical studies Jordheim and co-workers concluded that "it has been clearly shown that hENT1 expression is a predictive marker for patient outcome after (adjuvant) gemcitabine therapy" in resectable PDA[51]. This conclusion is also supported by the recently reported translational hENT1 data from the ESPAC studies: a retrospective subgroup analysis on 352 patients with resected PDA treated with either adjuvant 5-FU or gemcitabine found that hENT1 serves as a predictive marker for the efficacy of gemcitabine but not for 5-FU[52]. To overcome the poor prognosis in low hENT1 expressing PDA, CO-101, a chemically modified gemcitabine molecule thought to be capable of entering the cell independent of hENT1, was developed[53]. In

the 'low hENT1 and adenocarcinoma of the pancreas (LEAP)' trial, the efficacy and safety of CO-101 was investigated in chemo-naive metastatic PDA patients. Enrolling 360 patients, this international randomized phase II trial was also the first to prospectively assess the value of hENT1 as a predictive biomarker for treatment response to gemcitabine. Astonishingly, the LEAP trial not only demonstrated that CO-101 had no additional value over standard gemcitabine treatment, it also indicated that hENT1 expression does not correlate with overall survival in gemcitabine-treated patients with metastatic PDA[53]. Further research will have to elucidate whether these contrasting findings are due to different research approaches (retrospective *vs* prospective studies), differences in methodology (*e.g.* use of different antibodies) or if hENT1 expression has differing functions as a biomarker in the adjuvant and metastatic PDA setting.

RRM1 and dCK: Several other molecules involved in the metabolism of gemcitabine are currently under investigation as potential biomarkers in PDA: retrospective evidence suggests that - besides hENT1 - also deoxycytidine kinase (dCK) may be able to predict benefit from adjuvant gemcitabine in resected PDA[54]. dCK is responsible for the intracellular phosphorylation of the prodrug gemcitabine to its mononucleotide in a rate-limiting manner. Thus high expression levels of dCK may enhance the efficacy of the drug. RRM1 (ribonucleotide reductase M1) is a cellular target for gemcitabine and may additionally also act as a tumor suppressor. Preliminary evidence in resectable PDA showed that RRM1 may potentially serve as both a prognostic (in non-gemcitabine treated patients) and a predictive (in gemcitabine treated patients) biomarker[54].

Biomarker for the efficacy of nab-paclitaxel

SPARC: Adjacent stromal tissue is a hallmark of PDA believed to be an important contributor to poor treatment outcome by reducing drug delivery to cancer cells[55]. New treatment strategies aim on facilitating drug delivery to cancer cells by reducing tumor stroma. A promising candidate is the recently FDA-approved albumin bound nab-paclitaxel, which was originally developed to avoid toxicities observed in treatment with solvent-based paclitaxel[9]. In addition to a favorable safety profile, nab-paclitaxel has been shown to deplete tumor stroma and increase intratumoral gemcitabine concentration by a factor of 2.8 in mice bearing xenograft PDA tumors[56]. Further, co-administration of gemcitabine and nab-paclitaxel reduced levels of the gemcitabine metabolizing enzyme cytidine deaminase, making PDA cells more sensitive to gemcitabine treatment[9]. Recent results from the phase III 'metastatic pancreatic adenocarcinoma clinical (MPACT) trial showed a statistically significant increase in overall survival from 6.7 mo in patients receiving single-agent gemcitabine to 8.5 mo in patients receiving the combined nab-paclitaxel/gemcitabine regimen[57]. Recent findings in humans confirmed that

stromal depletion by nab-paclitaxel might be responsible for the reported survival benefit[58]. For intracellular uptake of nab-paclitaxel into PDA stromal cells, specific albumin-binding proteins are necessary. Secreted protein acidic and rich in cysteine (SPARC) has been proposed to be one of them[9]. SPARC is a matricellular glycoprotein involved in different biological processes like wound repair or angiogenesis[59]. Its overexpression and correlation with poor prognosis independent of the therapeutic agent has been described in different human cancers like colon, esophageal, breast and lung cancer[60]. In PDA it was demonstrated that increased SPARC expression in adjacent fibroblasts but not in cancer cells conversely correlates to overall survival[60]. Results from a phase I / II nab-paclitaxel trial suggested that elevated SPARC levels in fibroblasts adjacent to PDA might be a predictive marker for treatment success with nab-paclitaxel[56,61]. Yet a very recent study using SPARC knockout mice reported drug delivery and antitumoral effects of murine nab-paclitaxel to be independent of SPARC expression[62]. Thus further research will be necessary to elucidate the potential use of SPARC as a predictive biomarker for nab-paclitaxel treatment in humans; specifically the translational results on SPARC from the large international MPACT study are urgently awaited in this context.

Future directions

Translational research studies conducted so far have failed to identify reliable prognostic or predictive biomarkers for PDA. Besides methodological limitations like retrospective design and heterogeneous study populations, most trials focused only on specific mutations in a small number of genes. Yet prognosis and treatment response might depend on the interaction of a large variety of genes and mutations as proposed in a work of Collisson *et al*[63]. In this study microdissected DNA of resected PDA was analyzed using gene expression microarray analysis. A 62-gene signature for PDA was defined by means of different statistic models. Subsequently tumor probes were divided into three different subgroups depending on their genetic signature. Subgroups were classical type for PDA expressing high levels of adhesion-associated and epithelial genes, quasi-mesenchymal type for PDA expressing high levels of mesenchyme-associated genes and exocrine-like type for PDA expressing high levels of tumor cell derived digestive enzyme genes. Prognosis between these three subtypes differed significantly with classical type having the best and quasi-mesenchymal type having the worst prognosis regarding overall survival. In further experiments Collisson *et al*[63] analyzed human and murine PDA cell lines using the 62-gene microarray technique. Dependence on KRAS was analyzed using RNAi. Proliferation of classical subtype PDA cell lines was more prone to inactivation of KRAS by RNAi (of note this approach did not distinguish between wildtype and mutant KRAS alleles). Additionally, classical PDA cell lines were more sensitive to treatment with erlotinib while quasi-mesenchymal cell lines were more sensitive

to gemcitabine[63]. Further clinical research is required to translate these findings into clinical practice. As cancer genome sequencing becomes more available and less expensive[64], analyzing large subsets of genes appears to be a promising future approach to predict treatment response and prognosis in PDA.

CONCLUSION

In this topic highlight several potential biomarkers for prognosis, tumor biology and treatment response of PDA were identified and discussed (as summarized within Table 1). Despite promising pre-liminary results, translational research has failed to establish reliable biomarkers for clinical practice so far. Main limitations for most trials on potential biomarkers conducted in PDA were: non-comparable patient cohorts, retrospective design and non-consistent treatment protocols and molecular methods used. Besides the general need for more accompanying translational studies in pancreatic cancer trials, future studies on potential biomarkers should be conducted prospectively in well-defined patient populations, using standardized molecular methods and profound biostatistical analysis. Furthermore, innovative technologies like cancer genome sequencing and multiplex ELISA platforms might help to identify new options in predicting prognosis and facilitating treatment allocation in PDA.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Genetic predisposition to pancreatic cancer

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Abstract

Pancreatic adenocarcinoma (PC) is the most deadly of the common cancers. Owing to its rapid progression and almost certain fatal outcome, identifying individuals at risk and detecting early lesions are crucial to improve outcome. Genetic risk factors are believed to play a major role. Approximately 10% of PC is estimated to have familial inheritance. Several germline mutations have been found to be involved in hereditary forms of PC, including both familial PC (FPC) and PC as one of the manifestations of a hereditary cancer syndrome or other hereditary conditions. Although most of the susceptibility genes for FPC have yet to be identified, next-generation sequencing studies are likely to provide important insights. The risk of PC in FPC is sufficiently high to recommend screening of high-risk individuals; thus, defining such individuals appropriately is the key. Candidate genes have been described and patients considered for screening programs under research protocols should first be tested for presence of germline mutations in the BRCA2, PALB2 and ATM genes. In specific PC populations, including in Italy, hereditary cancer predisposition genes such as CDKN2A also explain a considerable fraction of FPC.

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Key words: Pancreatic adenocarcinoma; Susceptibility genes; CDKN2A; Melanoma; Hereditary cancer syndromes; Screening

Core tip: Pancreatic adenocarcinoma is the most deadly of the common cancers. Identifying families with hereditary pancreatic cancer can aid appropriate selection of individuals who are at high risk and are good candidates for prevention and screening programs. Although genetic predisposition to pancreatic cancer remains largely unexplained, next-generation sequencing is likely to provide important insights. Candidate genes have been described and patients considered for screening protocols should first be tested for germline mutations in these genes. In specific pancreatic cancer populations, including Italy, hereditary cancer predisposition genes such as CDKN2A also explain a considerable fraction of hereditary pancreatic cancers.

INTRODUCTION

Pancreatic adenocarcinoma (PC) is the deadliest among the common cancers. Its incidence is on the rise, especially in North America, Japan and Europe, where it represents the fourth to fifth most frequent cause of cancer mortality. Despite advances in therapy, diagnostic imaging and understanding of genetic factors, PC mortality rates have not declined appreciably in the past 20 years, and PC mortality still nearly equals its incidence (roughly

280000 new cases per year, 7000 of which are in Italy), leading to an estimated 227000 deaths per year worldwide[1-4]. The only potentially curative treatment for PC is surgical resection. Median survival following resection ranges from 13 to 21 mo, while without surgery, median survival is a mere 2.5-8 mo[5,6]. However, as most PCs are diagnosed late, and < 5% of tumors are resectable at the time of diagnosis, 5-year survival for PC remains low (< 5%). Early detection of stage 1 disease with curative resection has been shown to improve 5-year survival rates upwards to 60%[7].

The incidence of PC in the general population is not as high as that of other more common cancers (*e.g.*, colorectal cancer), therefore, nonselective screening is not recommended. However, targeted screening may hold promise for high-risk individuals (HRIs) identified by their family history or because of a known genetic predisposition. To date, no standard diagnostic approach or early detection method for PC has been developed, and screening remains challenging[8]. Accurate risk stratification and correct identification of HRIs with a genetic predisposition to the disease who may benefit from prevention and screening interventions in high-volume centers with ongoing research programs on PC[8,9] is thus crucial.

In recent years some excellent reviews have described susceptibility genes for PC, its biology and screening intervention protocols[8-12]. The aim of this review is to provide an update of recent findings on genetic susceptibility to PC, describing standard and novel approaches for the identification of susceptibility genes, as well as genetic data recently obtained for the first time in the Italian PC population.

RISK FACTORS FOR PANCREATIC ADENOCARCINOMA

Biological, lifestyle and environmental risk factors

PC incidence shows wide variations across countries, suggesting that biological, lifestyle and environmental factors are involved in determining increased PC risks, which range between two- and 13-fold[4]. PC is age dependent; in the United States, the median age at diagnosis is 72 years. Only 5%-10% of patients with PC develop the disease before the age of 50 years, and these are likely to include patients with an underlying genetic predisposition or who have previously undergone radiotherapy[4]. Sex and race also play a role, probably related to differences in smoking rates in men, as well as race-specific genetic differences in the ability to detoxify tobacco products, or vitamin D deficiency in blacks. Although these factors cannot be modified, lifestyle and environmental risk factors are controllable, cause 20%-25% of all PCs, and are thus of importance for HRIs. Heavy alcohol intake is associated with a modest increased risk of PC, while chronic pancreatitis, long-term diabetes, *Helicobacter pylori* infection, overweight, vitamin D deficiency and occupational exposures are associated with significantly increased risk. Conversely, atopic allergy and

use of metformin to treat diabetes have been associated with a reduced risk.

Susceptibility genes in HRIs

Although lifestyle modifications are possible and may help reduce PC risk, high-risk factors are not controllable and are the ones that typically characterize candidates for prevention and screening interventions.

PC has a familial basis in as many as 10% of patients. Some of the familial aggregation of PC is due to chance, and some to shared environmental exposure such as cigarette smoking[6]. An inherited predisposition to PC is seen in a range of clinical settings. Several hereditary cancer syndromes are known to be associated with an increased risk of PC, mainly Peutz-Jeghers syndrome (PJS), melanoma pancreatic-cancer syndrome (MPCS) or familial atypical multiple mole melanoma (FAMMM)-PC, hereditary breast-ovarian cancer (HBOC), and to a lesser extent Lynch syndrome (LS) and familial adenomatous polyposis (FAP). In addition, an increased risk of PC is present in patients with hereditary pancreatitis or cystic fibrosis.

Approximately 20% of hereditary cases of PC are currently attributed to a known genetic syndrome. The term familial pancreatic cancer (FPC) applies to the remaining 80% of patients with an inherited predisposition: families in which at least two first-degree relatives (FDRs) have been diagnosed with PC but that do not meet the diagnostic criteria for the previous settings[13-16] (Table 1).

Genes for known hereditary cancer syndromes

As mentioned earlier, PC is known to occur in a range of hereditary diseases and syndromes.

PJS is an autosomal dominant hereditary disease with characteristic hamartoma polyps of the gastrointestinal tract, and mucocutaneous melanin pigmentation. Almost half of all PJS patients harbor germline STK11/LKB1 gene mutations. Affected individuals have a 36% cumulative lifetime risk of developing PC[17].

FAMMM is an autosomal dominant disease that is characterized by the occurrence of > 50 atypical nevi and malignant melanoma in two or more first- or second-degree relatives. Malignant melanoma, however, may also be familial in the absence of the FAMMM phenotype. Approximately 10% of melanomas have a familial aggregation pattern and mutations in the CDKN2A tumor suppressor gene are identified in roughly 40% of these families[18]. PC has been observed in a considerable proportion of kindreds with CDKN2A mutations. This is considered to be a distinct hereditary cancer syndrome, is termed FAMMM-PC or MPCS, and has been found to confer a 17% cumulative lifetime risk of developing PC. CDKN2A germline mutations account for 30%-40% of patients affected by MPCS or the FAMMM-PC syndrome[18-29].

HBOC is another autosomal dominant hereditary cancer syndrome and is caused by germline mutations in

Table 1 Syndromes and genes associated with hereditary predisposition to pancreatic adenocarcinoma, relative and lifetime risk			
Settings of hereditary PC	**RR of PC (-fold)**	**Cumulative lifetime risk by age 70 (%)**	**Genes identified**
FPC syndrome			PALLD, CDKN2A, BRCA2, PALB2, ATM,....?
FDR with PC	2-3	2	
FDRs with PC	6	8-12	
or more FDRs with PC	14-32	40	
Hereditary cancer syndromes			
PJS	132	36	STK11/LKB1
MPCS/FAMMM	13-47	17	CDKN2A
HBOC	3.5-10	3-8	BRCA1, BRCA2
LS	8.6	< 5	MLH1, MSH2, MSH6
FAP	2-3	< 5	APC
Syndromes of chronic inflammation			
HP	50-80	40	PRSS1, SPINK1
CF	5	< 5	CFTR

HP: Hereditary pancreatitis; FAP: Familial adenomatous polyposis; PC: Pancreatic adenocarcinoma; FDR: First-degree relative; PJS: Peutz-Jeghers syndrome; MPCS: Melanoma pancreatic-cancer syndrome; FAMMM: Familial atypical multiple mole melanoma; HBOC: Hereditary breast-ovarian cancer; LS: Lynch syndrome; FPC: Familial PC; CF: Cystic fibrosis; HP: Hereditary pancreatitis.

the BRCA1 and BRCA2 genes. BRCA2 mutation carriers have an increased risk of breast, ovarian, and prostate cancer, as well as a 3.5-10-fold increased risk of PC[30,31], while the reported risk of PC for BRCA1 mutation carriers is about 2.5 times that of the normal population[32].

PC is also typical of LS, alternatively termed hereditary non-polyposis colorectal carcinoma syndrome. This syndrome is caused by mutations in the mismatch repair (MMR) genes MSH2, MLH1, MSH6 and PMS2. Individuals with mutations in the MMR genes have a risk of developing PC that ranges between 5% and 10%[33]. According to a recent study[34], PC risk is increased sevenfold in both MLH1 and MSH2 carriers belonging to LS families, especially at young ages, as noted by Lynch *et al*[35] as early as 1991.

Patients with FAP also have an increased risk of developing PC, with a relative risk of 4.6 (95%CI: 1.2-11.4)[36,37]. Finally, PC also occurs, if less frequently, in patients affected by Li-Fraumeni syndrome and ataxia telangiectasia.

Hereditary cancer syndromes in Italian PC patients

One of the difficulties in confirming that PC is a component of an inherited syndrome caused by germline mutations in a susceptibility gene is the lack of DNA from PC patients in families, which makes it impossible to conduct co-segregation analysis.

We recently investigated the contribution of hereditary cancer syndromes to PC in a hospital-based series of 225 Italian PC patients who were consecutively recruited at our center. Among these patients, 24% of those who presented with features suggestive of HBOC were BRCA1 or BRCA2 positive, and 10% of those who were suspected to be affected by LS carried mutations in the MMR genes[38,39]. Interestingly, 45% of the cases suspected for MPCS were found to harbor mutations in CDKN2A[40-42]. This result corroborates previous findings on the high occurrence of PC in Italian melanoma families with CDKN2A mutations[27,43-46]. The presence of CDKN2A mutations in PC patients selected from a case-control series

shows that an unbiased association exists between PC and CDKN2A germline mutations. No other hereditary syndromes were observed in this series that could drive selective screening of other genes.

Genes for hereditary conditions associated with PC

Hereditary pancreatitis: Hereditary pancreatitis (HP) is currently considered to be an independent nosological unit. It is an autosomally dominant disease with 80% penetrance. In patients with HP, trypsin becomes activated while still in the pancreas. This leads to partial digestion of the pancreatic tissue, causing inflammation.

A strong genetic association exists between HP and germline mutations in the PRSS1, SPINK1 and CFTR genes[47]. Patients with HP have an about 80% relative risk and a 40% lifetime risk of developing PC. If these individuals are smokers, then PC develops, or rather is diagnosed, up to two decades earlier than in non-smokers. Similarly, alcohol consumption also leads to a 20-year earlier diagnosis of PC[48,49].

Cystic fibrosis: Cystic fibrosis (CF) is an autosomal recessive disease that is caused by mutations in the *CFTR* gene. CF is characterized by the production of viscous mucus, which blocks the airways and leads to obstruction of the pancreatic duct, thus increasing the risk of inflammation. Patients with CF are at increased risk of chronic pancreatitis and of PC[50].

FPC genes

FPC is mostly inherited in an autosomal dominant fashion, and presents with a heterogeneous phenotype. Prospective studies have reported an increased risk of developing PC in unaffected FDRs of PC patients, which depends on the number of relatives with PC in the family[51]. This risk has been estimated to be 6.4-fold greater in individuals with two FDRs with PC (lifetime risk 8%-12%) and 32-fold greater in individuals with three or more FDRs with PC (lifetime risk 40%) (Table 1). Among kindreds with FPC,

the risk is higher in kindreds with young-onset PC (age < 50 years, relative risk = 9.3) compared with kindreds without young-onset PC[15,52,53]. Furthermore, evidence indicates that the risk of PC is modestly increased in FDRs of patients with sporadic PC compared to the general population[53], in which the lifetime risk of developing PC is slightly less than 1% (0.5% at age 70 years). Anticipation has been described in 59%-85% of FPC families; indeed, patients in younger generations are affected by the disease about 10 years earlier than their affected relatives[54,55].

Studies focusing specifically on FPC genes have not been successful so far in clarifying the genetic basis of the disease[8,15,52]. Several genes underlying susceptibility to the cancer syndromes associated with PC have been investigated for their involvement in FPC predisposition. Although the genes responsible for PJS[56] and LS[57,58] do not seem to play a major role, BRCA2 and BRCA1 are interesting candidates.

BRCA2 has been considered an important PC predisposition gene since its discovery[59], and recent reports have estimated that it accounts for 6%-12% of FPC families[8,60,61]. BRCA1 gene mutations have been reported in a small number of patients with FPC[62,63]. Increasing evidence is emerging that points to CDKN2A as an FPC susceptibility gene[42,64] and other, novel candidate genes (and loci) are being discovered. Indeed, over the past decade, FPC families have been found to harbor mutations in several different genes.

PALLD: In 2002, linkage analysis of a large FPC pedigree from the United States showed significant linkage to chromosome 4q32-34[65]. Four years later, an oncogenic germline mutation at this locus, in the Palladin (*PALLD*) gene, which encodes a cytoskeletal protein, was identified in affected members of that family. It was therefore suggested that PALLD may be a major PC susceptibility gene[66]. But this hypothesis was not supported by later studies on Italian FPC families and on families from other European countries[42,67,68].

BRCA1 and BRCA2: Although germline mutations in the *BRCA1* gene have been reported in a small number of patients with FPC[62,63], mutations in BRCA2 have long been reported to be the most frequently identified genetic alterations in FPC. Early studies with small sample sizes found BRCA2 mutations in 15% of FPC families from Germany and the United Kingdom and in 17% of families from North America[59,60]. These results however could not be confirmed in larger cohorts, in which deleterious BRCA2 mutations were detected in 6% of moderate- and high-risk FPC families[58,61]. BRCA2 deficiency in PC seems to be of clinical importance, because PCs in BRCA2-positive patients are characterized by marked sensitivity to poly (ADP-ribose) polymerase inhibitors and mitomycin[69-71].

We recently assessed the role of BRCA1 and BRCA2 as FPC susceptibility genes in the Italian population[42]

and found no germline mutations.

PALB2: PALB2, which binds to the BRCA2 protein, was reported to be a new PC susceptibility gene after whole genome sequencing identified truncating PALB2 mutations in 3.1% of a series of North American FPC patients[72]. PALB2 mutations were later detected in 3.7% of German and British FPC families[73]. Conversely, a Dutch study on 28 FPC families identified no mutations in PALB2[74]. These findings suggest that PALB2 mutations may explain FPC in a small subset of European families, especially in those with an additional occurrence of breast cancer. Indeed, PALB2 is increasingly considered a good candidate for clinical testing in BRCA1- and BRCA2-negative HBOC families[75]. PALB2 testing in a series of Italian PC patients suspected for HBOC described above, and in FPC patients, yielded no mutations, despite the fact that we screened the gene both by Sanger sequencing and by multiplex ligation-dependent probe amplification assay, in order to rule out large genomic rearrangements[38,42].

Germline mutations in other genes in the BRCA2 pathway, namely FANC-C and FANC-G, have been linked to early-onset PC, but segregating germline mutations in these genes have yet to be identified in FPC families[76].

ATM: Recently, heterozygous germline mutations in the ataxia telangiectasia mutated (ATM) gene have been identified in two kindreds with FPC[77]. Subsequent analysis of 166 additional FPC patients identified another four deleterious ATM germline mutations, while none were detected in 190 spouse controls. The prevalence of ATM mutations in the whole FPC cohort was 2.4% (4/166), and 4.6% (4/87) in families with three or more affected members[77]. These findings suggest that ATM mutations in these families may underlie PC, driven by the classic two-hit model for tumor suppressor genes.

CDKN2A: CDKN2A germline mutations account for 30%-40% of patients with MPCS or FAMMM-PC[18-29] and have generally been considered to play a minor role in FPC[61,78-80]. However, there is increasing evidence that CDKN2A (p16INK4a) mutations occur in FPC without metachronous or synchronous occurrence of melanoma in the family.

Two recent papers describing our Italian and the Dutch PC population suggest that CDKN2A may be an FPC susceptibility gene and that CDKN2A testing may be appropriate in FPC even when melanoma does not occur in the family[42,64]. Previously, a large North American study of 1537 unselected patients with PC found that 0.6% carried CDKN2A mutations. Among the 120 FPC cases in that study, four (3.3%) were CDKN2A positive. The authors concluded that screening of patients with PC for CDKN2A mutations should not be performed, but also that these mutations are especially penetrant among smokers[80].

Most CDKN2A mutations are missense mutations lo-

Table 2 Role of CDKN2A mutations in familial pancreatic adenocarcinoma and melanoma pancreatic-cancer syndrome

Study[1]	N° of FPC families	CDKN2A mutation found	Type of CDKN2A mutations	% of CDKN2A positive	N° of MPCS	CDKN2A mutation found	% of CDKN2A positive	Type of CDKN2A mutations
Slater et al[61] 2010; Bartsch et al[79] 2002	56	0	-	-	5	2	40	p.Q50X, p.E119X
McWilliams et al[80] 2010	119	3	c.-34G>T,p.V95fs, p.D153spl,	2.5	39	2	5.3	p.D153spl, pL16R
Ghiorzo et al[42] 2012	16	5	p.E27X,p.G67R, p.G101W, c. 201ACTC>CTTT	31	5	2	40	p.L65P, p.G101W
[2]Harinck et al[64] 2012	24	3	p.Ser8fs, p.Ala76fs	12	4	3	75	p.Ala76fs

[1]This table only includes studies that analyze CDKN2A mutations in pancreatic adenocarcinoma (PC) probands from familial PC (FPC) families comparing them with PC probands from Melanoma pancreatic-cancer syndrome (MPCS) families belonging to the same population. The prevalence of CDKN2A mutations in MPCS/FAMMM-PC e families, as analyzed in melanoma probands, is described in the text. [2]A melanoma was diagnosed in one FPC family after the proband was found to carry a mutation in CDKN2A.

cated in the coding sequences of exons 1 and 2, common to both the tumor suppressors encoded by this locus (p16INK4a and p14ARF). A number of these mutations seem to derive from ancestral founders[81]. We previously performed germline testing of CDKN2A in a series of unselected PC patients and found that 4% of these patients were CDKN2A positive[40,41]. In a subsequent study we extended the analysis to 225 PC patients and controls. The CDKN2A mutation rate in the 225 PC cases was 5.7%, ranging from 2.6% in patients without a family history of PC or melanoma, to 17% when two cancers occurred in the index patient or FDRs, and to 45% when three or more cancers occurred. Interestingly, 25% of the cases with one FDR with melanoma were mutation positive. Sixteen probands of FPC families were identified, defined for having at least two FDRs affected by PC, and no other manifestation of a hereditary cancer syndrome, or melanoma. Deleterious or potentially deleterious CDKN2A mutations were found in five of the probands (31%)[42]. The mutation frequency ranged from 20% in FPC families with two affected members to 50% in families with three, and was comparable to the mutation rate in melanoma families[46] (Table 2).

Within the PC families with no CDKN2A mutations, anticipation was observed, which is consistent with previous studies that reported anticipation for BRCA2 carriers in FPC families without CDKN2A mutations[55].

The CDKN2A mutation rate in our FPC cases was nearly 10 times that observed in the North American study by McWilliams and colleagues[80]. This result indicates that a sizeable subset of Italian FPC families may carry CDKN2A mutations, and likely reflects the prevalence of founder mutations in CDKN2A in our population[43-46,82,83].

Approaches to CDKN2A genetic testing

It has been proposed that individuals should be referred for CDKN2A testing when at least one of the following conditions is met: (1) a personal history of melanoma and an FDR with melanoma; (2) more than two confirmed primary melanomas; (3) more than three (first-degree or second-degree) relatives with melanoma; (4) a personal or a family history of PC and melanoma; (5) a personal history of melanoma; and (6) a personal and/or a family history of atypical moles[13]. Other recommendations have included patients with more than three melanomas, or families with at least one melanoma and two other instances of melanoma or PC in the family, with mutation yields ranging between 20% and 40%[84].

Had we followed these criteria we would have identified two out of five (40%) of our mutation-positive families with both melanoma and PC. However, as none of the criteria include FPC families, we would not have identified the CDKN2A-positive FPC kindreds. The North-American study mentioned earlier came to the same conclusion, because the majority of their mutations were identified in FPC families, despite their low overall mutation frequency[80]. Their finding is probably more generalizable than ours, both because of their sample size and because it was not influenced by the presence of founder mutations.

Taken together, our results confirm that the occurrence of at least three cancer events (including PC and melanoma) in the family is a good predictor of CDKN2A mutations (45%). Importantly, however, the likelihood of identifying a CDKN2A mutation may also be high in families with two or more instances of PC or with one instance of PC and one of melanoma among FDRs, because we found that 17% of such kindreds were positive for CDKN2A mutations[42].

Harinck and colleagues also performed CDKN2A mutation analysis in 28 FPC families. Unlike ours, their selection criteria included presence of melanoma, and indeed melanoma also occurred in four of their families (14%), Interestingly, CDKN2A mutations were identified in three of these melanoma-positive families, confirming that CDKN2A mutations are frequently found in families affected by both PC and melanoma. The prevalence of CDKN2A mutations in their FPC families with no occurrences of melanoma was 12% (n = 3). These CDKN2A-positive families would not have been identified had the recommendations mentioned above

been followed, which are based on studies that found no CDKN2A mutations in FPC families without melanoma[13,26,61,79]. The prevalence of CDKN2A mutations in the FPC families studied by Harinck et al[64] may have actually been underestimated. Indeed, affected relatives in some of the families in that study were unavailable for DNA testing, so unaffected FDRs were tested instead. In such cases a negative test does not rule out the presence of a pathogenic mutation unless a specific mutation has been found in another relative.

Harinck and colleagues concluded that CDKN2A mutations are found in a considerable proportion of families with FPC (Table 2), and therefore CDKN2A mutation analysis should be performed in FPC families even in the absence of reported melanomas. According to the authors this strategy will enhance the recognition of individuals at risk for PC and facilitate the early detection of melanomas.

A number of reports have suggested that BRCA2 mutation analysis should be performed in FPC families that do not meet the criteria for HBOC[59,61]. Similarly, our findings and those reported by Harinck and colleagues emphasize the need to include CDKN2A mutation analysis in genetic testing for FPC families, even in the absence of reported melanomas[42,64].

Novel predisposition genes: evidence from NGS and genome-wide association studie

The discovery of additional FPC genes is one of the most exciting opportunities in PC research. As the speed and ease of testing increase and costs fall as a result of NGS, we expect that a number of new FPC genes will be discovered in the coming years. Exome sequencing has already led to the identification of PALB2 and ATM mutations in FPC, and much hope is being placed in postgenomic studies[72,77]. Indeed, recent genome-wide association studie (GWAS) and post- GWAS analyses have identified chromosome regions containing novel susceptibility loci for PC.

One such study, the PanScan Project, has identified several common polymorphisms affecting PC susceptibility. In that study, single nucleotide polymorphisms (SNPs) in ABO, sonic hedgehog (SHH), telomerase reverse transcriptase, nuclear receptor subfamily 5, group A, member 2 were found to be associated with PC risk. The scan also identified loci on chromosomes 13q22.1 and 15q14, to which no known genes or other functional elements are mapped[85,86].

Another GWAS on PC risk has been performed in the Japanese population[87], and yielded three new loci on chromosomes 6p25.3 (SNP rs9502893, 25 kb upstream of FOXQ1), 12p11.21 (SNP rs708224, in the second intron of BICD1) and 7q36.2 (SNP rs6464375, in the first intron of DPP6). Another still has been conducted in the Chinese population[88] and identified five novel PC susceptibility loci at chromosomes 21q21.3 (SNPrs372883, in the 3' UTR of gene BACH1), 5p13.1 (SNP rs2255280, in intron 1 of gene DAB2), 21q22.3 (SNP rs1547374,

upstream of gene TFF1), 22q13.32 (SNP rs5768709) and 10q26.11 (SNPrs12413624). The latter two SNPs are not located in the immediate vicinity of any gene.

Several recent reports have also shown associations between other genetic variants and PC risk and progression, and their impact on survival is currently being investigated[89-91].

The ABO gene in particular has been further investigated, and a link between ABO blood type and PC has been established. Non-O blood types have been found to account for 17% of all new PC cases, showing a protective effect of the O blood group. However, the exact mechanism that links PC and blood group remains unclear[92]. Whether genetic variability at the ABO locus may be involved in PC survival is currently under investigation[93].

Recent analysis of GWAS data has revealed that two pathways, the neuroactive ligand receptor interaction and olfactory transduction, are significantly associated with PC risk, and has shown that four genes are significantly associated with PC risk, adding OR13C4 to the previously identified ABO, HNF1A and SHH[2-4] genes. These findings provide new insights into the polygenic basis of PC susceptibility and etiology[94].

Gene-environment interaction

Among PC families, the risk of developing PC is higher in younger subjects and is likely modified by nongenetic risk factors such as exposure to tobacco smoke. Not only do smokers have a 2-3-fold greater risk of developing the disease compared to non-smokers, but they generally develop the disease at an earlier age[95,96].

An interesting example of gene-environment interaction for PC was shown for germline CDKN2A mutations in the large North American hospital-based study mentioned earlier, which investigated both the prevalence of germline mutations in PC patients, and their penetrance[80]. The authors found that penetrance for PC and melanoma was increased among mutation carriers, with PC risk estimates of 58% (95%CI: 8%-86%) by age 80 years and melanoma risk estimates of 39% (95%CI: 0%-80%) by age 80 years. Among ever-smokers, the risk of PC was higher for CDKN2A mutation carriers compared to non-carriers (HR = 25.8, $P = 2.1 \times 10^{-3}$), but among non-smokers the comparison did not reach statistical significance. The authors concluded that CDKN2A mutations in PC patients are rare but notably penetrant, and that CDKN2A mutation carriers, as well as being candidates for prevention and screening studies, should be counseled to avoid tobacco use.

Identification of HRIs: in silico analyses, genetic testing, role of registries

Genetic testing can identify a family's underlying genetic susceptibility to PC, but has limited scope because the genetic basis of much of the inherited susceptibility to this disease remains unexplained. Additional PC susceptibility genes may be discovered in the near future that should improve our ability to identify individuals who

Table 3 Proposed inclusion criteria for pancreatic adenocarcinoma screening programs in high-risk individuals, identified based on family history and possibly on genetic background

Current (based on family history alone or on genetic background):
  Family history:
    Three or more relatives in the same lineage affected by PC
    Two relatives affected by PC, at least one of which is a FDR of the individual
    Hereditary pancreatitis
    > 10-fold increased risk as established by PancPRO
  Genetic background:
    Germline carrier of a mutation in a candidate gene with at least one FDR or SDR affected by PC
    Mutation-positive individual in a PJS kindred
Proposed (based on family history and genetic background):
  Family history: Identification of a hereditary syndrome or a 10-fold increased risk established by PancPRO
  Genetic background: According to testing in candidate genes (CDKN2A, BRCA1-2, ATM, PALB2, STK11, PRSS1, SPINK1…)
    Mutation identified: Propose screening to carriers of germline mutation
    No mutation identified: Propose screening to all HRIs
In populations with a high prevalence of germline mutations in candidate genes (e.g., CDKN2A founder mutations in Italy or the Netherlands)
The same as above + test candidate genes according to specific genetic background, even in the absence of all criteria for hereditary syndromes or of a PancPRO score > 10

PC: Pancreatic adenocarcinoma; FDR: First-degree relative; PJS: Peutz-Jeghers syndrome; HRIs: High-risk individuals.

would benefit most from pancreatic screening in the context of research protocols[11].

Family history remains the main tool to quantify PC risk. Risk stratification is determined by the number of affected individuals in the family and the degree of relatedness between those individuals and other family members. The phenotypic variance seen in FPC families and the heterogeneity of the hereditary cancer syndromes potentially involved require careful study of the family tree over at least three generations, and histopathological confirmation of all diagnoses.

A computer-based risk assessment tool, PancPRO (http://astor.som.jhmi.edu/BayesMendel/pancpro.html), which uses this type of information has been shown to provide an approximate risk assessment for FPC families[97,98]. Families with high PancPRO scores would generally be identified by standard criteria, but PancPRO has the advantage that it can assign a quantitative risk score to any family member, which also depends on the age at diagnosis (or death) of the affected relatives. PancPRO provides useful information about an individual's PC risk before he or she decides to undergo invasive screening. That information can also help identify appropriate candidates for research on screening protocols or genetic susceptibility. Indeed, according to a recent position paper by the Italian PC Registry, having a PancPRO risk score > 10 is one of the criteria for enrollment in screening programs for PC[99].

It is in the framework of these programs that our findings will be of value to establish the most appropriate criteria to select families for CDKN2A testing in Italy. We found that about 30% of our FPC patients with no occurrence of melanoma among relatives carried mutations in CDKN2A, and similar results have been reported in the Netherlands, therefore, we suggest that individuals considered at high risk because of their family history should undergo genetic testing for CDKN2A before they are enrolled in research surveillance programs,

especially in populations such as these, in which founder effect CDKN2A mutations are predominant (Table 3).

Genetic testing for hereditary PC mandates full informed consent as recommended by national guidelines for genetic testing for cancer susceptibility[100] and should be initially performed in affected individuals[11,13]. Germline genetic testing of patients with PC is currently underused, not least because clinicians often fail to take a detailed history of cancer occurrence in the family. The possibility that a hereditary cancer syndrome may be present in the family is therefore frequently overlooked. However, our data show that a considerable proportion of FPC families carry CDKN2A mutations, even in the absence of melanoma in the family.

A combination of risk prediction tool analysis and genetic testing is likely to be the most successful approach to identify HRIs (Table 3). Based on the results reviewed here, genetic testing should be performed after PancPRO analysis to stratify better risk and identify the HRIs who may benefit from PC surveillance programs performed in the context of research protocols.

More generally, affected members of FPC families should be analyzed for BRCA2, PALB2, ATM and CDKN2A mutations. Genetic analyses of other genes (e.g., LKB1 and BRCA1) should only be recommended if the family history is suggestive of the associated hereditary cancer syndromes[11].

CONCLUSION

PC remains one of the most challenging of all cancers. Numerous studies are currently under way to identify novel early detection tools for PC, and evidence is beginning to show that screening FDRs of individuals with several family members affected by PC can identify precursors of this malignant disease[8-13].

Prospective PC screening with endoscopic ultrasound, magnetic resonance imaging and magnetic resonance

cholangiopancreatography has been shown to detect precancerous lesions with a diagnostic yield ranging from 13%[101] to 76%[102], depending on study population (high or moderate risk, carriers or non-carriers of germline mutations), age at baseline screening, screening modalities, and definition of the diagnostic yield, with the highest yield obtained in confirmed carriers of CDKN2A germline mutations[103].

Appropriate inclusion of families at high risk of PC in registries provides an excellent tool to improve our clinical and genetic understanding of FPC[104]. Indeed, focused research projects can be conducted most efficiently when data from different FPC registries are combined.

Although much work is currently focused on clarifying the impact of common genetic variability on individual PC risk, much less is known about heritable susceptibility to PC compared to what is known about other heritable cancers. One viable option to expand our understanding of the genetic determinants of PC risk is to collect large sets of patients across different populations[91]. In this review we have described some important results on new susceptibility genes and loci that have been recently obtained by PC consortia[10].

Genetic risk factors are believed to play a major role, and several germline mutations have been identified that underlie hereditary susceptibility to PC in different settings, such as FPC and other hereditary cancer or chronic inflammation syndromes. The risk of PC in FPC is sufficiently high to recommend screening HRIs; therefore defining those HRIs appropriately is crucial.

In the general population, the lifetime risk of developing PC is 1%. Although they have a twofold increased risk of PC, the vast majority of individuals with a family history of PC will not develop the disease themselves. It is therefore important to explain the concepts of both relative and absolute risk to patients and their families. However, when an FDR of a PC patient is tested and found to carry a germline mutation in a high-risk gene, the risk is not negligible. Once penetrance and factors that modify penetrance have been taken into account, these individuals may be appropriate candidates for prevention or screening protocols, which should at all events only be directed at HRIs, defined to the best of our ability and possibly with genotypic data.

Utility analyses suggest that PC screening is most cost-effective in individuals whose lifetime risk of the disease is 16% or greater[9]. It can detect intraductal papillary mucinous neoplasm and pancreatic intraepithelial neoplasia, which are precursor lesions for FPC; importantly, the former are higher grade, more common, and multifocal in individuals with FPC compared with patients with sporadic PC[9].

In this review we emphasize the importance of testing CDKN2A in Italian patients with hereditary PC, even when there is no occurrence of melanoma in the family, in order to improve the accuracy of risk stratification and ensure appropriate selection of patients, which we think may be especially of value in populations with a high CD-

KN2A mutation rate (Table 3). Identifying high-risk family members is important to understand the biology of PC, to recommend risk reduction strategies and, in some cases, enrollment in cancer surveillance programs. Because the best methods for surveillance have yet to be established and given the overall complexities involved, HRIs and FPCs should be referred to, screened and managed by multidisciplinary teams with specific experience, in the context of research protocols at high-volume centers.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Cancer stem cells: Involvement in pancreatic cancer pathogenesis and perspectives on cancer therapeutics

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Abstract

Pancreatic cancer is one of the most aggressive and lethal malignancies. Despite remarkable progress in understanding pancreatic carcinogenesis at the molecular level, as well as progress in new therapeutic approaches, pancreatic cancer remains a disease with a dismal prognosis. Among the mechanisms responsible for drug resistance, the most relevant are changes in individual genes or signaling pathways and the presence of highly resistant cancer stem cells (CSCs). In pancreatic cancer, CSCs represent 0.2%-0.8% of pancreatic cancer cells and are considered to be responsible for tumor growth, invasion, metastasis and recurrence. CSCs have been extensively studied as of late to identify specific surface markers to ensure reliable sorting and for signaling pathways identified to play a pivotal role in CSC self-renewal. Involvement of CSCs in pancreatic cancer pathogenesis has also highlighted these cells as the preferential targets for therapy. The present review is an update of the results in two main fields of research in pancreatic cancer, pathogenesis and therapy, focused on the narrow perspective of CSCs.

Key words: Cancer stem cells; Pancreatic cancer; Cancer stem cells signaling pathways; Targeted therapy; miRNA

Core tip: Pancreatic cancer is one of the most aggressive and lethal malignancies, despite remarkable progress in understanding pancreatic carcinogenesis and new therapeutic approaches. The presence of highly resistant cancer stem cells (CSCs) and the changes in their signaling pathways lead to drug resistance in pancreatic cancer. CSCs are considered responsible for tumor growth, invasion, metastasis and recurrence. CSC involvement in pancreatic cancer pathogenesis has also highlighted them as preferential targets for therapy. This review is an update of the results in two main fields of research in pancreatic cancer, pathogenesis and therapy, focused on the narrow perspective of CSCs.

Tanase CP, Neagu AI, Necula LG, Mambet C, Enciu AM, Calenic B, Cruceru ML, Albulescu R. Cancer stem cells: Involvement in pancreatic cancer pathogenesis and perspectives on cancer

INTRODUCTION

Pancreatic cancer is one of the most aggressive and lethal malignancies. Despite remarkable progress in understanding pancreatic carcinogenesis at the molecular level as the identification of new therapeutic approaches, pancreatic cancer remains a disease with a dismal prognosis; the five-year survival rate is approximately 5%[1]. Although several histological subtypes of pancreatic cancer have been described, the most common form is pancreatic ductal adenocarcinoma. According to data published by the International Agency for Research on Cancer, pancreatic cancer death is the eighth or ninth most frequent cause of cancer death worldwide and is the fourth or fifth most common cause of cancer death in developed countries[2,3].

The main risk factors for pancreatic cancer include increasing age, smoking[4], chronic pancreatitis, diabetes mellitus, metabolic syndrome, low levels of serum vitamin D, family history of pancreatic cancer and rare inherited genetic conditions such as Peutz-Jeghers syndrome, familial melanoma and hereditary pancreatitis[5]. Age is a significant risk factor; the median age at diagnosis is 72 years. Pancreatic tumors are rarely diagnosed before the age of 50, and such cases are very likely to be associated with underlying predisposing genetic disorders. Approximately 5%-10% of pancreatic cancer patients report a family history of pancreatic cancer. The genes responsible for a minority of the familial clustering of pancreatic cancer have been identified, including STK11, CDKN2A, PRSS1, BRCA2 and PALB2[1,6].

The high mortality rate of pancreatic cancer is due to difficulty in early diagnosis[7,8] and its notorious resistance to chemotherapy and radiation[9]. Lack of clinical symptoms in early stages leads to delay in tumor detection; thus, approximately 80% of patients with pancreatic cancer have metastatic disease at the moment of diagnosis[10]. Existing systemic therapies for advanced disease are far from effective, and the median survival for patients with metastatic disease remains 6 mo. Surgery offers a better prognosis of a cure, but even those patients who undergo resection and receive adjuvant therapy have a median survival of 12-22 mo and a 5-year survival of 20%-25%[2,11].

Chemoresistance is a critical issue in pancreatic cancer. Among mechanisms responsible for drug resistance, the most relevant are changes in individual genes or signaling pathways, the influence exerted by tumor microenvironment (desmoplastic reaction) and the presence of highly resistant cancer stem cells (CSCs)[9]. The notion of CSCs has gained prominence, and several identified molecules and signaling pathways are relevant for the diagnosis and therapy of cancer. The paradigm of cancer-initiating stem cells has initially been developed with respect to blood cancers, where chronic conditions such as myeloproliferative neoplasms are due to mutations acquired in hematopoietic stem cells[12].

CANCER STEM CELLS IN PANCREATIC CANCER PATHOGENESIS

Cancer stem cells involvement in tumorigenesis

Pancreatic cancer (especially pancreatic ductal adenocarcinoma) is an aggressive malignancy, with one of the worst prognoses among solid tumors. Pancreatic cancer is typically diagnosed in late stages, when most patients are inoperable and when curable treatment is not available. Current therapies (radio- and chemotherapy) may improve prognosis and reduce tumor size but cannot target all pancreatic cancer cells[13,14].

Cancer stem cells, identified in a large number of human malignancies, represent 0.2%-0.8% of pancreatic cancer cells and are considered responsible for tumor growth, invasion, metastasis and recurrence[15]. Currently, there are two models that explain tumor development[16-18]. The stochastic model states that every cancer cell has the ability to initiate and promote tumoral growth. The other model, the "cancer stem cell hypothesis", proposes that tumor evolution is based on stem cells with a 'deregulated' self-renewal pathway. A recent and rapidly growing body of research shows solid evidence in support of the cancer stem cell model against the stochastic model[19,20]. The American Association for Cancer Research defines CSCs as cells within a tumor that have the capacity to generate the heterogeneous cancer cell lineages found in the tumor and that possesses the capacity to self-renew. CSCs also share other several important attributes: active telomerase expression, drug resistance to harming agents, the activation of antiapoptotic pathways, the ability to migrate and to metastasize and increased membrane transporter activity. To date, CSCs have been isolated and characterized only from a relatively small number of tumor types: breast, brain, pancreas, colon, blood and head and neck[21,22]. Several studies argue that cancer stem cells cannot be eradicated by current therapy and thus are responsible for tumor relapse and metastasis[23]. Many studies have demonstrated that multiple critical genes, including K-ras, p53 and p16, and key signaling kinases, such as PI-3K, mTOR, NF-κB, epidermal growth factor receptor (EGFR) and SHH, play important roles in pancreatic tumorigenesis[24].

Pancreatic cancer stem cells markers

Several pancreatic cancer stem cell (PCSC) subpopulations have been isolated using flow cytometry and combinations of positive and/or negative membrane surface markers[25-29]. Historically, research on stem cells and cancer stem cells from the hematopoietic system began long before studies in other tissues. As a result, several

markers identified in hematopoietic malignancies, such as cluster of differentiation (CDs), were also proposed as potential PCSCs markers. Li *et al*[30] were the first to identify a population of PCSCs using CD44, CD24 and ESA as separation markers. The cell fraction with the CD44+/CD24+/ESA+ phenotype exhibits several important cancer stem cell characteristics, including a minor population of cells (between 0.2% and 0.8%) that has the potential to form tumors in half of the mice used for transplantation. *In vitro* studies lend further support to arguments for the use of CD44 and CD24 as cancer stem cell markers. CD44+/CD24- cells isolated from PANC-1, a pancreatic adenocarcinoma cell line, exhibit a much higher tumorigenic potential than cellular subpopulations not expressing the markers[31]. Prominin-1 or CD133 is another important marker used for isolating PCSCs. Hermann *et al*[32] demonstrated that CD133+ cells form more tumors than CD133- populations. Another important finding of the study is that cells positive for CD133 and for CXCR4 exhibit a higher metastatic potential than other populations from the same tumors, supporting the observation that CXCR4 may be involved in tumor invasion and metastasis. A recent study provided further evidence for the role of CXCR4 in pancreatic cancer, demonstrating that human pancreatic ductal adenocarcinomas contain a side population of cells with CSC properties and high expression levels of CXCR4 and ABCB1[33]. Moreover, these genes correlate with poor patient survival rates. c-Met is a hepatocyte tyrosine kinase growth factor upregulated by CD44[34]. C-Met was also shown to be a PCSC marker[35,36]. Interestingly, cells expressing c-Met have the same tumor-forming potential as CD44+/CD24+/ESA+. Furthermore, CD133+/c-Met-high are less tumorigenic than CD44+/c-Met-high[35]. Aldehyde dehydrogenase 1 is another marker expressed by cancer stem cells. Studies report that ALDH1 can identify PCSCs and protect the tumor pancreatic cells from programmed cell death induced by radiotherapy[35,37]. Other studies demonstrate that pancreatic cancer stem cells are characterized by genetic and epigenetic alterations associated with carcinogenesis and can form xenograft tumors in immunodeficient mice[38,39].

Limitations of the current methods for isolating cancer stem cells from pancreatic cancer include the lack of specific PCSC markers and the needed to understand the molecular mechanisms that regulate the specific biological properties of PCSCs.

Another important line of research focuses on biomarkers that regulate PCSC properties and behavior[40]. Thus, nestin can modulate important characteristics of PCSCs, such as invasion or metastasis, and may represent a viable target for anticancer therapy. A recent study Lu *et al*[41] reported that Oct 4 and Nanog play important roles in pancreatic cancer by regulating PCSC behavior and suggested that these molecules may represent prognosis markers. Both CD44+/CD24+/ESA+ and pancreatic tumor CD133+ subpopulations are characterized by the overexpression of Nanog, Oct4, Notch1,

MDR1 and ABCG2 and are capable of metastasizing to distant sites, such as the liver[33,42]. Moreover, inhibiting their expression impairs PCSC characteristics. Other reports demonstrate that markers such as DCLK1 can discriminate between normal and tumoral stem cells and that knockdown of DCLK1 decreases molecular pathways that control pancreatic tumorigenesis. Another important regulator of stem-like characteristics in PCSCs is SOX2, which controls cellular proliferation and differentiation[43]. C-kit with KRAS were also proven to modulate the progression of pancreatic adenocarcinoma, supporting the assumption that the use of drugs that downregulate the activity of these markers can improve the prognostic of the disease[44].

One of the main causes of high mortality in this pathology is the resistance to chemotherapy, which is also believed to be mediated by cancer stem cells within the tumor mass[45,46]. In 2013, Lu *et al*[41] demonstrated that in the pancreatic cancer cell line PANC-1, the highly expressed stem cell markers Oct4 and Nanog are associated with chemoresistance, proliferation, migration, invasion, and tumorigenesis *in vitro* and *in vivo*. This study also indicated the potential use of these two transcription factors as prognostic markers and targeted therapies in pancreatic cancer. Another study in a murine model reported that the ALDH+ and CD44+CD24+ cell populations are resistant to treatment with gemcitabine, one of the main chemotherapeutic agents[47].

Shah *et al*[48] has developed a gemcitabine-resistant cell line that exhibits higher expression of the pancreatic CSC markers CD44, CD24, and c-Met, which are also associated with epithelial-mesenchymal transition (EMT).

Aldehyde dehydrogenase (ALDH), considered to be a marker for cancer stem cells, is a detoxification enzyme with increased activity in many cancer types where its presence has been associated with decreased survival[49]. An *in vitro* study revealed that ALDH expression is correlated with the invasiveness of pancreatic cancer cell lines and that patients with ALDH-positive tumors have poor prognosis[49].

It is unclear what the initial molecular events underlying the conversion of tissue stem cells to cancer stem cells in pancreatic cancer; some studies suggest that appearance of c-kit and KRAS mutations might be the primary events in the initial stages of this disease and have proposed c-kit as a potential therapeutic target[44]. Almost all pancreatic cancers are characterized by activating mutations in KRAS and the loss of p16INK4A, but these cancers are also characterized by mutations in the tumor suppressors SMAD4 and p53. More studies suggest the involvement of these genetic alterations in the development of cancer stem cell properties and surface marker profiles.

Another theory suggests that EMT is responsible for the appearance of cells with stem cell-like properties that are characterized by the activation of many pathways involved in EMT[27]. EMT is a crucial process for tumor progression, involving the transformation of epithelial
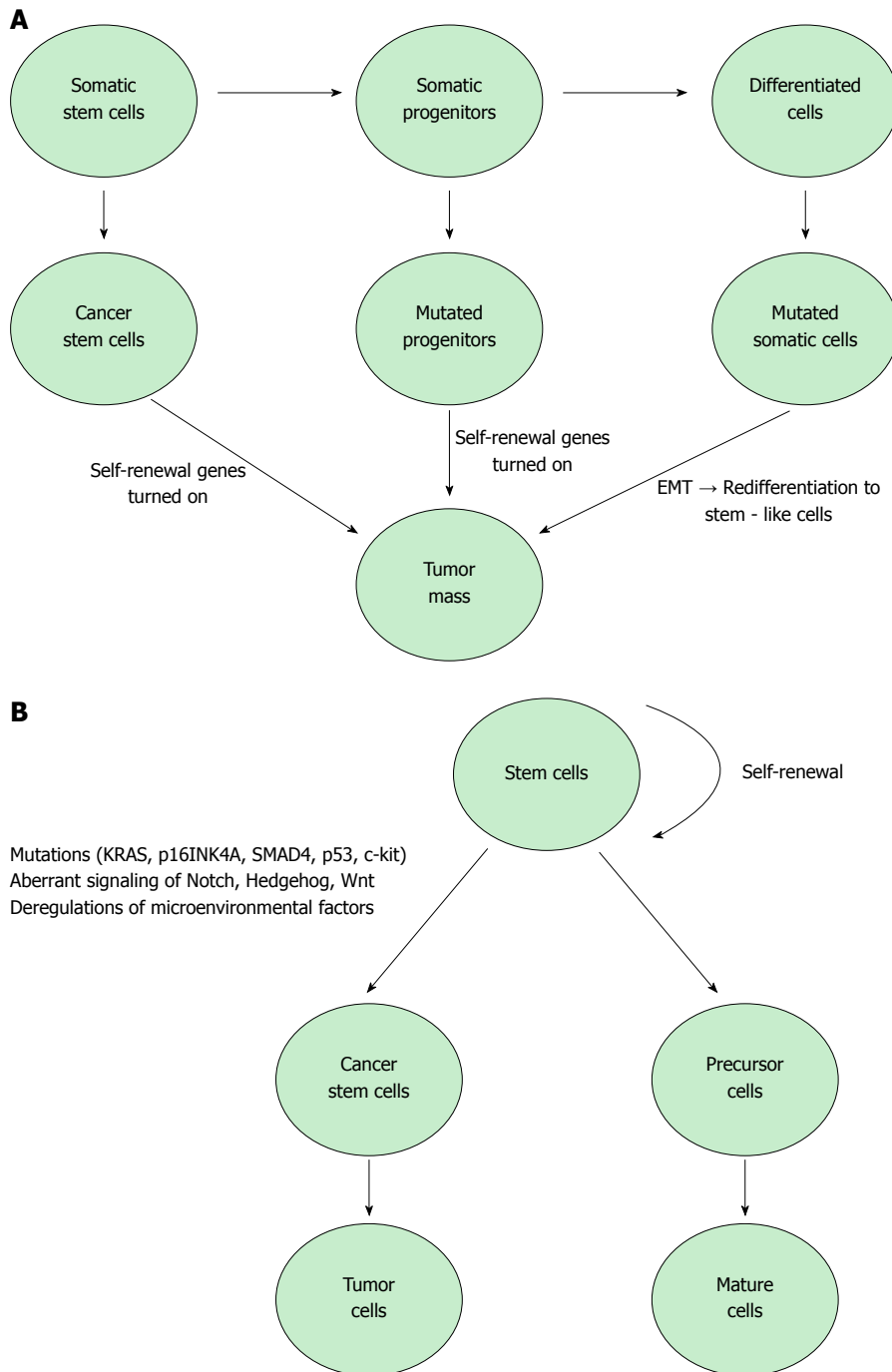
Figure 1 Models that explain tumor development. A: The stochastic model states that every cell has the potential to be the "the cell of origin" of a tumor; B: The "cancer stem cell hypothesis" proposes that tumor evolution is based on stem cells with 'deregulated' signaling pathways. EMT: Epithelial-mesenchymal transition.

characteristics into mesenchymal characteristics, which subsequently allow cancer cells to disseminate from the tumor mass[50].

Signaling pathways involved in pancreatic cancer stem cells

Several signaling pathways are altered in CSCs and EMT-like cells in pancreatic cancer: Hedgehog, Notch, Wnt, AKT and NF-κB (Figure 1). Hedgehog, Notch and Wnt have been shown to be of particular importance in pancreatic cancer stem cells, due to their role in pancreatic

embryonic development and differentiation[51]. These signaling pathways play important roles in the self-renewal of CSCs, tumor growth, invasion, metastasis and resistance to therapy[27]. MiRNAs were recently considered to play a role in the regulation of CSCs[15].

Notch signaling is involved in cell proliferation, survival, apoptosis and the differentiation of pancreatic cells and can promote EMT by controlling some transcription factors and growth factors like Snail, Slug, and TGF-β. Some of the Notch target genes are Akt, cyclin D1, c-myc, COX-2 (cyclooxygenase-2), ERK (extracel-

lular signal-regulated kinase), MMP-9 (matrix metallo-proteinase-9), mTOR (mammalian target of rapamycin), NF-κB (nuclear factor-kappa B), VEGF (vascular endothelial growth factor), p21cip1, p27kip1, and p53, all involved in the development and progression of human cancer. Gemcitabine-resistant pancreatic cancer cells exhibit overexpression of Notch-2 and Jagged-1, whereas Notch1, a key downstream mediator of KRAS, is responsible for pancreatic sphere formation[15,28,52]. Many studies found that pancreatic cancer stem cells resistance to chemotherapy is linked to activated Notch signaling, but the exact mechanism remains unclear[9,53]. There is more evidence detailing that the Notch signaling pathway is essential in supporting the ability of KRAS to transform normal cells into tumor stem cells. In this regard, in pancreatic cancer treatment, Notch signaling inhibition can be more attractive, as long as there are no data arguing that Notch signaling has a critical role in normal adult pancreas homeostasis[54].

Hedgehog signaling is another self-renewal pathway allowing normal stem cells to become independent of control signals; as a result of mutations in this signaling, transformed cells can use Hedgehog for tumor initiation, progression and metastasis. *In vivo* studies revealed that compared with normal pancreatic epithelial cells, CD44+CD24+ESA+ pancreatic cancer stem cells exhibit up-regulation of Shh transcripts, the ligand of Hedgehog signaling[55]. Moreover, 70% of pancreatic cancer tissue exhibits overexpression of Shh, suggesting that Hedgehog signaling may be involved in pancreatic carcinogenesis[15]. Studies in the pancreatic cancer cell line PANC-1 demonstrated that the inhibition of Hedgehog signaling by SMO suppression can reverse EMT, induce apoptosis *via* PI3K/AKT inhibition and inhibit the invasion of pancreatic cancer cells[56]. Moreover, a combination of focal irradiation with Hedgehog signaling inhibition reduces lymph node metastasis in an orthotopic animal model[57].

Wnt/β-catenin signaling is involved in cell proliferation, migration, apoptosis, differentiation, and stem cell self-renewal in several types of cancers[58]. Wnt/β-catenin signaling pathway dysregulation is also associated with chemoresistance in pancreatic cancer, and recent studies suggest that nuclear β-catenin is essential for EMT[50,59]. *In vitro* and *in vivo* studies suggest that activated β-catenin may decrease the differentiation of epidermal stem cells, increase self-renewal capacity and promote epithelial cancers in transgenic mice[60].

In 2013, Sun *et al*[38] reported that one of the most activated signaling pathways in pancreatic cancer stem cells is NF-κB, whose inhibition leads to loss of stem cell properties. This study also indicated that aberrant epigenetic processes, like CpG promoter methylation, can be involved in carcinogenesis mediated by cancer stem cells.

Cancer stem cells from epithelial tissues were identified for the first time in breast cancer in 2003, when Al-Hajj *et al*[61] reported that a distinct population CD44+CD24-/low ESA+ develops tumors in immunodeficient mice.

In pancreatic cancer, the presence of cancer stem cells was reported in 2007 by Shah *et al*[48] who showed that CD44+CD24+ESA+ cells exhibit high tumorigenic potential.

MicroRNAs in pancreatic adenocarcinoma

As often found in many cancers, expression of miRNAs appears to be dysregulated in pancreatic cancer. The miRNA complement of cancer cells appears different than that in normal tissue.

MiRNAs are potent regulators of cell function *via* their role as translational regulators for the synthesis of key proteins. Most often, several miRNAs exhibit different expression profiles in cancer cells.

MiR-21, miR-155 and miR-17-5p appear upregulated in tumoral cells, and these miRs are often called oncogenic miRNAs[62,63]. Similarly, a series of miRNAs, referred to as tumor suppressor miRs miR-34, miR-15a, miR-16-1 and let-7), are downregulated in cancers[64,65].

Key cell differentiation programs during development are controlled by the members of the let-7 and miR-200 families. In cancer, the loss of let-7 leads to disease progression and de-differentiation. The same let-7 family appears as a regulator of EMT and of stem cell maintenance. The EMT process is regulated by miRNA-dependent mechanisms. In human pancreatic cancer, DCLK1 regulates EMT by a mechanism dependent on miR-200a[66,67].

According to Haselmann *et al*[65], the inhibition of the maturation of let-7 by nuclear TRAILR2 in pancreatic cancer cell lines increases their proliferation. This result is consistent with high levels of nuclear TRAIL2 in tissue samples from poor outcome patients.

The population of BxPC-3-LN cells (lymph node metastatic pancreatic cells) contains a 5-fold increased population of CD133+/CXCR4+ cells (stem cell-like cells) compared with the parental (non-metastatic) BxPC-3 cells. Remarkably, a different miRNA pattern is exhibited in CSC-like cells compared with the non-CSC-like cells: up-regulated miR-572, miR-206, miR-449a, miR-489 and miR184 were observed in conjunction with downregulated let-7g-3p, let-7i-3p, let-7a-3p, miR-107, miR-128 and miR-141-5p[68].

The miR-200 family members have been identified as key regulators of cell maintenance and EMT. Tumor progression may represent progressive de-differentiation (EMT) towards a cell type having a stem cell-like phenotype. This process appears to be regulated by miRNA-dependent mechanisms. DCLK1 (a putative marker for pancreatic and intestinal cancer stem cells) regulates EMT in human pancreatic cancer cells *via* a miR-200a-dependent mechanism[69]; DCLK1 also acts as a regulator of let-7a in pancreatic and colorectal cancer cells, supporting the concept that these miRNAs may be novel and relevant targets in solid tumor cancers[63,70]. Sureban *et al*[23] demonstrated that DCLK1 inhibition results in the up-regulation of miRNAs that negatively regulate some key angiogenic and pluripotency factors. In AsPC1

tumor xenografts, the downregulation of c-MYC and KRAS *via* let-7a was observed in a mechanism similar to that demonstrated in pancreatic cancer cells.

The repression of two tumor-suppressor miRs, miR-143 and miR-145, is reported in pancreatic cancer as well in other cancers[71]; moreover, experimental restoration of miR 143/145 levels using nano-vector delivery was demonstrated to inhibit pancreatic cancer cell growth[72]. The miR-143/145 cluster cooperates and inhibits the expression of KRAS2 and RREB1, its downstream effector[71]. MiR-145 was demonstrated to inhibit cell proliferation in lung adenocarcinoma by targeting EGFR. In many cancers, including pancreatic cancer, EGFR is upregulated[73], whereas inhibition of EGF signaling inhibits cancer initiation and progression[74]. Furthermore, a suppressive effect of EGFR on miR-143 and miR-145 was demonstrated in models of colon cancer[75]. These findings are indicators of a negative feedback loop between EGFR and miR-143/145, which is similar to KRAS/RREB1-miR-143/145.

The major role of VEGF signaling *via* its receptors, VEGFR1 and VEGFR2, was demonstrated in tumor vascular growth, angiogenesis, and metastasis, and upregulated angiogenic factors in various cancers (colorectal, breast, renal, liver, and ovarian) have been correlated with poor prognosis. PDAC exhibits endothelial cell proliferation, a mechanisms that increases angiogenesis. Inhibition of VEGF-A, VEGFR1 and VEGFR2 resulted in the inhibition of tumor growth and angiogenesis in mouse models of PDAC. Studies and computational analysis outlined a putative binding site for miR-200 (miR-200a, b and c) in the 3' UTR of VEGFR1 and VEGFR2[76].

More studies suggest that stem cells convert to cancer stem cells by the deregulation of miRNA expression, which affect several signaling pathways involved in proliferation, apoptosis, and more importantly, renewal and differentiation of stem cells[77,78]. Nanog and Sox2, important regulators of stem cell pluripotency, and the CD44 stem cell surface marker are examples of these miRNAs targets[79].

Using microarray analysis, Jung *et al*[70] demonstrated that pancreatic cancer stem cells exhibit differential expression of miR-99a, miR-100, miR-125b, miR-192, and miR-429 compared with controls. An in vitro study conducted on the human pancreatic cancer cell lines AsPC-1, AsPC-1-GTR, MiaPaCa-2, and MiaPaCa-2-GTR revealed re-expression of miRNAs (let-7a, let-7b, miR-26a, miR-101, miR-200b, and miR-200c) that are normally lost in pancreatic cancer and especially in pancreatic spheres can revert or destroy CSCs[80]. Another study reports the loss of miR-34 in CD44+CD133+ MiaPaCa2 pancreatic cancer cells, whereas miR-34 restoration led to the inhibition of a side cell population of tumor cell sphere growth and of tumor formation[64].

Wellner *et al*[71] demonstrated that miR-200c, miR-203 and miR-183 activity can lead to the downregulation of stem cell factors, founding a regulatory feedback loop between miRNAs and CSC in pancreatic cancer.

In this regard, an understanding of miRNAs alterations can lead to the development of better strategies in the treatment of pancreatic cancer patients by the elimination of CSCs.

The identification of dysregulated miR expression and the existence of regulatory loops between miRs and protein regulators of key processes (such as cell growth, angiogenesis, differentiation) suggest the need and potential effectiveness of strategies aiming to restore the "normal phenotype" expression pattern of miRs for cancer treatment. Various approaches have been developed and investigated, such as the delivery of tumor suppressor miRs[81,82], the suppression of expression or action of oncomirs[83,84], targeting the expression of key regulators (such as DCLK1, AMPKα1)[23,85] leading to miR modulation or the simultaneous modulation of multiple miRs, suggesting that using miRs as therapeutic agents or addressing miRNAs as targets represents a potential solution for the therapy of critical cancers.

CANCER STEM CELLS AS THERAPEUTIC TARGETS IN PANCREATIC CANCER

In pancreatic cancer, surgery is usually accompanied by other complementary treatments such as multi-chemotherapy regimens and radiotherapy. Despite clear progress in the detection and treatment of cancer, current strategies fail to completely remove the tumor and prevent recurrence and metastasis. Existing therapies are toxic and nonspecific, being directed towards both normal cells and tumor cells. Most chemotherapeutic regimens are based on gemcitabine but provide a modest improvement in median survival. The response rate was increased by using more than two chemotherapeutic agents[86]. Therapy failure for highly malignant tumors has been explained, at least partially, by the chemo-[87,88] and radioresistant[89] nature of CSCs. Furthermore, studies have demonstrated that gemcitabine regimens, by targeting differentiated cancer cells, lead to a relative enrichment of cancer stem cells[47].

The resistance of CSCs has been explained by several mechanisms: (1) expression of multidrug resistance-linked genes, largely ATP-binding cassette (ABC) drug transporters[90]; (2) activation of Wnt/β-catenin signaling[91]; and (3) activation of Hedgehog pathway[92]. Hence, a series of strategies preferentially target CSCs.

Signaling pathway targeting: Monoclonal antibodies and small molecule kinase inhibitors

TGFβ-related inhibition abrogated the self-renewal capacity of CSCs and *in vivo* tumorigenicity and reversed the resistance of orthotopically engrafted cancer stem cells to gemcitabine. The study demonstrated that the tumor response is, however, limited by the stromal hindering of drug delivery. The addition of a stroma-targeting hedgehog pathway inhibitor enhanced the delivery of the Nodal/Activin inhibitor and translated into long-term, progression-free survival[93].

The *Hedgehog* signaling pathway is usually targeted in experimental designs as an adjuvant to classic chemotherapy. The combined blockade of sonic hedgehog and mTOR signaling together with gemcitabine is capable of eliminating pancreatic CSCs[94]. Inhibition of Smoothened combined with gemcitabine prolonged survival in mice transplanted with pancreatic tumors. Importantly, however, only in mice treated with triple therapy (with mTOR inhibitor rapamycin added) were cancer stem cells virtually completely abrogated, and the authors reported long-term disease stabilization or regression and subsequent long-term survival[95].

Targeting *stemness genes* (*Sox2*, *Oct4* and *c-Myc*) through a complex decoy oligonucleotide designed to simultaneously target all three genes was shown to suppresse CSC properties and phenotypes and minimized the tumorigenic capability of the SP cells and the resistance to chemotherapy[42].

Several studies have targeted the *Notch* pathway using selective γ-secretase inhibitors. In pancreatic cancer xenografts, PF-03084014, a selective γ-secretase inhibitor, alone and in combination with gemcitabine, inhibited the cleavage of the nuclear Notch 1 intracellular domain and Notch targets Hes-1 and Hey-1 and induced tumor regression in 3 of 4 subcutaneously implanted xenograft models. The authors argue that the observed effects are due to PF-03084014 targeting putative aggressvive cancer stem cells[54]. Another potent and selective γ-secretase inhibitor, MRK-003, also led to the downregulation of the nuclear Notch1 intracellular domain, the inhibition of anchorage-independent growth, and a reduction in the number of cells capable of extensive self-renewal. Pretreatment of a pancreatic adenocarcinoma cell line with MRK-003 significantly inhibited the subsequent engraftment in immunocompromised mice, and the mixed regimen MRK-003 and gemcitabine in engrafted mice reduced tumor cell proliferation and induced both apoptosis and intratumoral necrosis[96].

Due to their involvement in cell proliferation, receptor tyrosin-kinases are frequently dysregulated in cancers and have been recently therapeutically targeted by small molecule inhibitors. There are reports of pancreatic cancer trials testing both kinase inhibitors and monoclonal antibodies. Sunitinib targets multiple receptor tyrosine kinases, including stem cell factor receptor (c-KIT) and has been shown to possess antitumor efficacy in *in vivo*. The combination of gemcitabine with sunitinib could not surpass the effects of single-agent sunitinib[97]. Cabozantinib, a small kinase inhibitor that targets c-Met and VEGFR2, inhibited viability and spheroid formation and induced apoptosis in pancreatic malignant cells with minor effects in non-malignant cells. In primary, CSC-enriched spheroidal cultures, cabozantinib downregulated the CSC markers SOX2, c-Met and CD133 and induced apoptosis[98].

Tumor-necrosis factor family members have also been targeted as possible anticancer therapies through monoclonal antibodies. A combination of tigatuzumab, a fully humanized death receptor 5 agonist monoclonal antibody, with gemcitabine proved to be more efficacious in killing both CSCs and adenocarcinoma cells. The combination therapy produced a remarkable reduction in pancreatic CSCs, tumor remissions, and significant improvements in the time to tumor progression[99].

Cell cycle regulators represent another class of molecules with the potential to be used as targets in anticancer therapies. Thus, inhibiting checkpoint kinase 1 (Chk1), together with gemcitabine was shown to decrease the capacity of PCSCs to initiate tumors. Another interesting finding was that DNA damage mediated by Chk1 was lower in non-stem cells than in stem cells[100].

Immunotherapy

Given the failure of cytotoxic therapies, new therapy approaches are under investigation. Vaccination therapy aims to increase the patient's immune response against tumor cells by targeting cancer markers with the aid of specialized antigen-presenting cells such as dendritic cells. Currently, there is a number of vaccines for human pancreatic cancer in clinical trials including the following: (1) whole-cell vaccines; (2) combined dendritic cells with antigen to present to patient leukocytes; (3) peptide and DNA vaccines, iv) Ras peptide vaccine; (4) vaccine against common cancer mutations targetable by CD4/8 T cells; (5) telomerase peptide vaccine; (6) CEA and Mucin 1; and (7) survivin-targeted vaccine[101].

Furthermore, boosting the immune response with additional treatment with dendritic cells (LANEX-DC®) was shown to be highly effective and to extend the median survival times up to 8.9 mo[102].

A rather recent and innovative approach in immunotherapy is personalized peptide vaccination (PPV), in which HLA-matched peptides are selected and administered, based on the pre-existing host immunity before vaccination[103]. PPV is now under investigation for pancreatic adenocarcinoma, and a phase Ⅱ study for 41 chemotherapy-resistant advanced pancreatic cancer patients has been reported. Vaccine antigens were selected and administered based on the pre-existing IgG responses to 31 different pooled peptides, and no vaccine-related severe adverse events were observed[104].

Other active agents

Salinomycin is a antiprotozoal agent that was recently proven to preferentially kill breast CSCs[105] and was later investigated in other types of malignancies (reviewed in[106]). In an *in vitro* model of pancreatic adenocarcinoma, salinomycin inhibited the growth of CSCs, and *in vivo* xenografting studies demonstrated that salinomycin combined with gemcitabine could eliminate the engraftment of human pancreatic cancer more effectively than the individual agents[107]. The mechanisms proposed for the anti-tumor activity of salinomycin include the following: (1) inhibition of Wnt/β-catenin signaling[108]; (2) induction of apoptosis and autophagy *via* AMPK activation[108]; (3) increased DNA breakage and phos-

phorylated levels of p53 and H2AX[109]; and (4) cell cycle arrest and apoptosis *via* downregulation or inactivation of cell cycle-associated oncogenes, such as Stat3, cyclin D1, and Skp2[110]. Adamantyl-substituted retinoid-related molecules (ARRs) inhibit growth and induce apoptosis in the pancreatic stem-like cell population, possibly through decreased IGF-1R and β-catenin expression[111].

Isothiocyanate sulforaphane (SF) was used as sensitizer of pancreatic CSCs to TRAIL (tumor necrosis factor-related apoptosis inducing ligand)-induced apoptosis by quercetin and sorafenib. Combination of SF with a cytotoxic drug efficiently induced apoptosis along with the inhibition of self-renewing potential, ALDH1 activity, clonogenicity, xenograft growth and relapse of GEM-treated tumor cells in nude mice[112].

The flavonoid Quercetin enhances TRAIL-mediated apoptosis, acts as a chemosensitizer for the ABC pump-proteins and can enhance the effects of sulforaphane in inhibiting pancreatic CSC characteristics[113].

Delivery of cytotoxic drugs by specific targeting of stem cell markers

Targeted therapeutic delivery is a way to ensure that drugs reach the designated target at the highest concentration within safety limits. Targeted delivery relies on nanoparticles [small metallic or non-metallic molecules, (such as polymeric, carbonic, sillica-for a detailed review please see[114])]. Most nanoparticles accumulate in tumors due to their intense and leaky neovascularization, but some can be retained in the tumors with the use of cancer-specific antigens[115]. In the same manner that nanoparticles are targeted for the bulk tumor, nanoparticles can be targeted for CSCs by CD-133, for example. To increase delivery into the cytosol and prevent early lysosomal degradation, Bostad *et al*[116] have employed photochemical internalization (PCI), a minimally invasive method for light-controlled, specific delivery of membrane-impermeable macromolecules to increase the cytotoxic effect of an immunotoxin targeting CD133-expressing cancer cells of colon (WiDr and HCT116) and pancreas (BxPC-3) origin.

CONCLUSION

Pancreatic cancer remains one of the major causes of cancer death with low survival rates due to the metastasis of early-stage tumors and the lack of any effective treatment. Discoveries made in recent years clearly demonstrate that stem cells and EMT-type cells are involved in pancreatic cancer and are responsible for chemoresistance and the metastatic potential of this tumor type. The emergence of cancer stem cells is based on genetic alterations and modifications in signaling pathways that result in the transformation of normal stem cells, progenitors or differentiated cells. Currently, cancer stem cell inhibitors in combination with conventional therapy are being tested in clinical trials and could provide an innovative approach for the treatment of pancreatic cancer.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Prognostic factors related with survival in patients with pancreatic adenocarcinoma

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Abstract

The prognosis in patients with pancreatic cancer is poor and this cancer is the fourth leading cause of cancer-related death worldwide. Although surgical resection is the only curative treatment of choice for pancreatic cancer, the majority of patients are diagnosed at an advanced stage, thus only 10%-15% of them are suitable for curative resection and the overall survival is less than 5%. Chemotherapy for metastatic disease is to palliate symptoms of patients and to improve survival. Therefore, prognostic factors are important and a correct definition of poor prognostic factors may help to guide more aggressive adjuvant or aggressive treatment protocols in patients with pancreatic cancer. This article reviews the prognostic factors affecting survival of patients with pancreatic cancer in the light of recent advances in the literature.

Key words: Pancreatic cancer; Prognostic factors; Survival; Carbohydrate antigen 19-9; Treatment

Core tip: The overall prognosis associated with pancreatic cancer has not improved over the last 20 years, even if new diagnostic and therapeutic strategies have emerged. Thus, investigations on predictive factors in pancreatic cancer are needed because these factors should have predictive value in relation to longer survival after surgery than after palliative treatment. Prognostic factors are important and a correct definition of poor prognostic factors may help to guide more aggressive adjuvant or aggressive treatment protocols in patients with pancreatic cancer.

INTRODUCTION

Pancreatic adenocarcinoma still remains a major public health issue and is the fourth leading cause of cancer-related death worldwide[1]. Although surgical resection is the only curative treatment of choice for pancreatic cancer, unfortunately, the majority of patients are diagnosed at an advanced stage, and thus only 10%-15% of them are suitable for curative resection and the overall survival is less than 5%[2,3]. Chemotherapy is used in the adjuvant setting and in the treatment of locally advanced inoperable and metastatic disease.

The primary goals of chemotherapy for metastatic disease are palliation and improved survival[4,5]. Therefore, identifying poor prognostic factors that may predict the tumor recurrence and prognosis of patients is important for selecting appropriate treatment protocols. So it is important to determine new biological or pathological indicators related to survival in addition to well-

known prognostic factors such as clinical and pathological stage, performance status, and surgical margin[6]. In this article, the prognostic factors affecting survival of patients with pancreatic cancer were reviewed.

SURGICAL AND PATHOLOGICAL FACTORS

The primary surgical or pathological factors that influence prognosis are whether the tumor is localized at the pancreas and whether the tumor has spread to lymph nodes or distant organs[1] because the highest cure rate occurs if the tumor is truly localized to the pancreas. In the present TNM staging system, tumor size, peripancreatic extension, and vascular involvement are used. Traditionally, TNM staging, especially in the presence of metastasis (advanced stage), has been found to be an important prognostic factor in patients with pancreatic cancer for survival[7-9].

Surgical margin

Surgical resection is the only potentially curative option for treatment of pancreatic cancer and the nature of surgery for resectable tumors depends on the tumor localization and size. The incidence of R1 resection has been indicated as being 20% in the literature, but the improvement of pathological work-up procedures has increased the rate of R1 resection up to 80%[10,11]. Menon et al[12] reported that of 27 patients with pancreatic cancer, 22 patients underwent R1 resection and the median survival rate for patients with R1 resection was significantly worse than that of patients with R0 resection (14 mo vs not reached). In a study performed by Raut et al[13], they reported that the rate of R1 resection was 16.7% and patients who underwent an R1 resection had a median overall survival (OS) of 21.5 mo compared with 27.8 mo in patients who underwent an R0 resection. In addition, multivariate analysis showed that high mean operative blood loss and large tumor size were independent predictors of an R1 resection, but margin status did not independently influence survival.

Another study including 265 pancreatic carcinoma patients who had undergone surgical resection reported that R1 resection in 49 patients (51%) and R2 resection in four patients (4%) were performed[14]. The R1-positive margin was localized at the retroperitoneal resection margin in 76% and at the trans-section margin in 14% of tumors. Median survival time was better in R0-resected patients compared with R1-resected patients (22 mo vs 15 mo). A positive resection margin after pancreatic resection is considered to be a poor prognostic factor, and some have proposed that an R1 margin may be a biologic predictor of more aggressive disease. On the other hand, whether these patients with pancreatic carcinoma who underwent margin-positive resection have to be managed with aggressive treatment modalities has not been described.

Lymph nodes status and lymph node ratio

Lymph node ratio (LNR) may be more useful than nodal (N) status in prognostic subclassification of pancreatic adenocarcinomas after pancreatoduodenectomy. Recent studies have suggested that LNR may also be an important prognostic factor in pancreatic cancer[15-17]. In the TNM staging system, the number of resected lymph nodes may be very important, but node-positive patients are not a homogenous group, because stage migration may occur in resected pancreatic cancer patients. To resolve these limitations, recently LNR was proposed as a new prognostic factor by several authors to prevent the 'stage migration' phenomenon[15-17]. Riediger et al[17], in 204 resected patients, reported that LNR was the strongest predictor of survival and they concluded that the routine estimation of the LNR may be helpful not only for the individual prediction of prognosis but also for the indication of adjuvant therapy. The analysis of Surveillance, Epidemiology, and End Results and MGH (Massachusetts General Hospital) in 10254 and 827 resected patients, respectively, showed that higher LNR (> 0.2) was associated with worse survival by univariate analysis, and in addition the hazard ratio (HR) raised proportionally when more lymph nodes were examined in multivariate analysis. This study concluded that while the contribution of the number of positive nodes to survival was relatively small, LNR was strongly associated with survival, and thus, LNR provided a stronger and more accurate predictor of survival than the number of positive nodes[18].

Perineural and blood vessel invasion

Both perineural (PNI) and blood vessel invasion (BVI) have been previously investigated in patients with pancreatic cancer and found to be important prognostic indicators for survival[14,19,20]. Lee et al[19] showed that PNI was an important adverse prognostic factor for patients with surgical resection, as was pN stage. In a study performed by Chatterjee et al[21], PNI and BVI were found to be associated with the OS and lymph node status in patients who were treated with neoadjuvant treatment. The median OS for patients with PNI was worse than that of patients without PNI (22 mo vs 36 mo). Moreover, the median OS was better in patients without BVI compared with patients with BVI (34 mo vs 22 mo). They detected that retroperitoneal resection margin was correlated with the presence of both BVI and PNI. The authors concluded that PNI and BVI were significantly poor prognostic indicators.

Tumor localization

Some studies have investigated the prognostic significance of tumor localization in pancreatic cancer patients, but there is currently no consensus[7-9,19,22]. In a study performed by Park et al[8], univariate analysis indicated that tumor location was an important prognostic factor for

Table 1 Surgical and pathological factors in pancreatic cancer		
Ref.	No. of patients	Results
Surgical margin/resection (R1 vs R0)		
Menon et al[12]	27	mOS, 14 mo vs NR
Raut et al[13]	360	mOS, 21.5 mo vs 27.8 mo
Lymph nodes status and lymph node ratio		
Riediger et al[17]	204	LNR was an independent prognostic factor
Valsangkar et al[18]	14907	LNR was strongly correlated with survival
Perineural and blood vessel invasion		
Chatterjee et al[21]	86	mOS, 34 mo for BVI (-) vs 22 mo for BVI (+); mOS, 32 mo for PNI (-) vs 22 mo for PNI (+)
Tumor localization		
Park et al[8]	340	It was an important prognostic factor by univariate analysis
Zhang et al[7]	302	It was an independent prognostic indicator
Operative factors		
Nagai et al[23]	271	OBL greater than 2000 mL was an independent prognostic factor for OS
Keck et al[24]	270	PBT was an independent prognostic indicator for survival

mOS: Median overall survival; NR: Not reach; LNR: Lymph node ratio; BVI: Blood vessel invasion; PNI: Perineural invasion; OBL: Operative blood loss; PBT; Perioperative blood transfusion.

OS, but the significance of tumor site as an independent prognostic indicator could not be proved in the multivariate analysis. Lee et al[22] showed that high CEA level was significantly correlated with tumor location. In the patients with elevated CEA level, tumors were located mostly at the pancreas body and tail. The authors could not show that tumor location was a prognostic factor by multivariate analysis, although in the univariate analysis it was detected as being a prognostic factor. However, in another study carried out by Zhang et al[7], localization of the primary tumor was found to be an independent prognostic factor. In other words, the mortality risk was increased for tumors located at the body and tail of the pancreas compared to the tumors located at the head and neck of the pancreas.

Operative factors

An influence of operative blood loss (OBL) on survival in patients with pancreatic cancer after curative resection has been investigated. Nagai et al[23] retrospectively analyzed 271 patients and found that the OS was significantly affected by the amount of OBL. The median survival times were 26.0, 15.3, and 8.7 mo for OBL less than 1000, 1000 to 2000, and greater than 2000 mL, respectively (< 1000 mL vs 1000-2000 mL, $P = 0.019$; 1000-2000 mL vs > 2000 mL, $P < 0.0001$). Moreover, OBL greater than 2000 mL was also detected to be an independent prognostic factor in multivariate analysis

(HR = 2.55) and OBL of 2010 mL was found to be an appropriate cut-off level to predict early mortality within 6 mo after resection. Male sex, year of resection, and plexus invasion were independently associated with OBL greater than 2000 mL. In light of these results, the authors concluded that excessive OBL was found to be a prognostic determinant of survival and it can be used to stratify the risk for pancreatic cancer mortality after surgery for pancreatic cancer. On the other hand, prognostic significance of perioperative blood transfusion (PBT) has also been reported. In a study performed by Keck et al[24], PBTs were given in 46% of 270 pancreatic cancer patients. Univariate analysis showed that PBT was related with poorer survival, as were positive margins, more than one involved node, and poorer grading. In addition, they found that PBT was an independent prognostic indicator for survival by multivariate analysis after resection. The authors thought that impact of PBT was independent of the perioperative complications or resection type. Table 1 shows selected trials of surgical and pathological prognostic factors in pancreatic cancer.

CLINICAL FACTORS

Performance status

Some studies have evaluated the impact of performance status (PS) on survival for patients with pancreatic adenocarcinoma, but the results are conflicting. In a study carried out by Sezgin et al[25], the authors reported that only PS was an independent prognostic factor for OS in patients with advanced pancreatic cancer. Similarly, Tas et al[26] found that initial poor PS (PS 2-4) was significantly associated with worse survival for patients with all stages of pancreatic cancer. In addition, poor PS remained as an independent prognostic indicator for survival by multivariate analysis and in patients with poor PS, severe weight loss (> 10%), large tumor diameter (> 3 cm), and especially metastatic disease was related with significantly shorter OS. On the other hand, in another study, although an influence of PS on survival was detected in the univariate analysis, its prognostic significance was lost in multivariate analysis[8]. Lee et al[22] showed that in the elevated CA19-9 level group (≥ 37 U/mL), PS was significantly higher compared with the normal CA19-9 group. Furthermore, PS (0 vs 1-2) was found to be an important prognostic factor in the univariate analysis for OS.

Diabetes mellitus, obesity and jaundice

Diabetes mellitus (DM) is commonly diagnosed in pancreatic cancer patients, but the significance of new-onset DM as a cause of underlying pancreatic cancer is unknown. Some studies have investigated the prognostic significance of DM in pancreatic cancer[8,25,27], but an impact of DM on survival could not be proved.

Cachexia is a known characteristic of pancreatic cancer with detects as 80% of patients cachexic at diagnosis. Therefore, measurement of body mass index (BMI) at

the time of diagnosis does not provide accurate representation of a patient's long-term exposure to obesity[28]. However, some studies have shown that high BMI is associated with increased risk of pancreatic cancer incidence and mortality[29,30]. On the other hand, studies of obesity and survival in patients with pancreatic cancer are notably controversial. In a population-based study including 510 patients with pancreatic cancer, Gong et al[31] indicated that elevated HR of 1.3 was detected for obese (BMI ≥ 30) compared with normal range BMI (< 25) patients. But, the relation between OS and BMI could not be found. Similarly, recent study evaluated the association of BMI with the risk of death from pancreatic cancer in a pooled analysis of data from Asia Cohort Consortium[32]. It did not support an relation between BMI and risk of death from pancreatic cancer. As a different these studies, in a study carried out by Yuan et al[33] the association of prediagnostic BMI with pancreatic cancer survival was analyzed. Higher prediagnostic BMI was associated with more advanced stage at diagnosis, with 72.5% of obese patients presenting with metastatic disease versus 59.4% of healthy-weight patients. Furthermore, higher baseline BMI was associated with reduced survival. HR for death was 1.53, comparing BMI ≥ 35 kg/m^2 with BMI < 25 kg/m^2 ($P = 0.001$).

In a study performed by Smith et al[34], the presence of preoperative jaundice was found to be associated with poor survival in patients with pancreatic cancer. Another study showed that preoperative jaundice was the only independent prognostic factor for pancreatic cancer patients[19]. On the other hand, Perini et al[35] demonstrated that both preoperative DM and jaundice had no adverse effect on survival for curative resection in pancreatic cancer patients. Recently, Strasberg et al[36] analyzed 400 patients with resected pancreatic cancer, and preoperative jaundice was found to be a significant indicator of poor outcome in the multivariate analysis. Moreover, the relationship was detected between jaundice and nodal status, and jaundiced patients who underwent preoperative stenting had a survival advantage. The underlying mechanism related with the influence of jaundice on survival is unknown and additional studies are required to determine the exact mechanism for this effect.

Treatment and gemcitabine

Chemotherapy is only modestly effective in advanced disease but has a significant impact in the adjuvant setting, with 5-fluorouracil and gemcitabine both having efficacy in a subgroup of patients and increasing 5-year survival from 10%-15% with surgery alone to 20%-25%[37-40]. Park et al[8] analyzed 340 patients with pancreatic cancer and of 141 stage Ⅲ patients, 57 received supportive care (BSC) only, 25 received chemotherapy (CT), and 59 received concurrent chemoradiotherapy (CCRT); of the 199 stage Ⅳ patients, 119 were treated with BSC only and 80 received CT. Univariate analysis showed that CT and CCRT were significant prognostic indicators for OS in stage Ⅲ patients compared with patients that received

BSC only (11.3 mo vs 10.4 mo vs 6.4 mo, respectively; $P < 0.001$). Similarly, in stage Ⅳ patients, median OS for patients who were treated with CT was significantly better than that of patients who received BSC only (6.4 mo vs 3.1 mo, $P < 0.001$). In addition, initial treatment effect remained an independent prognostic factor compared to BSC only in the multivariate analysis[8].

In a study performed by Lee et al[19], gemcitabine chemotherapy was found to be the only independent prognostic indicator for OS in advanced or unresectable pancreatic cancer patients who had undergone palliative surgical by pass. Moreover, Zhang et al[7] evaluated 302 all-stage pancreatic cancer and found that the median OS of patients who did not receive any treatment or those treated with BSC only was 1.3 mo, while the median OS for patients who had undergone surgery, CT, biliary drainage therapy, arterial interventional CT, and comprehensive CT was 11.0, 7.3, 3.5, 9.0, and 11.0 mo, respectively ($P < 0.05$). In the multivariate analysis, the presence of treatment vs no therapy or BSC only was an independent prognostic factor (HR = 13.93, $P = 0.000$). However, platinum combination CT was significantly associated with improved OS compared to non-platinum CT regimen (HR = 0.56, $P = 0.011$). Selected trials related with clinical prognostic factors are summarized in Table 2.

LABORATORY AND MOLECULAR FACTORS

Prognostic role of carbohydrate antigen 19-9 levels

Serum carbohydrate antigen (CA) 19-9, the sialylated Lewis blood group antigen defined by the monoclonal antibody 1116 NS 19-9, is a tumor-associated antigen synthesized by normal pancreatic and ductal cells[41]. CA19-9 is considered to be the standard serum marker of pancreatic cancer due to its high sensitivity of 70%-90% and specificity of around 90%[42]. Serum CA19-9 levels have been found to be a useful tumor marker in differentiating benign from malignant pancreatic lesions, and to monitor tumor response to treatment[42,43]. Previous studies suggested that preoperative CA19-9 levels could predict the resectability of pancreatic cancer[44,45], and other studies reported that pretreatment CA19-9 level was an important prognostic factor in patients with pancreatic cancer who received CT or CCRT[8,9,45,46].

Park et al[8] reported that elevated CA19-9 levels (> 670 U/mL) were found to have prognostic significance for OS by univariate analysis, while it was an independent prognostic factor for OS in the multivariate analysis. Furthermore, another study found similar findings. The median OS time for patients with high CA19-9 level was worse than that of patients with normal CA19-9 level (3.8 vs 5.0 mo), which was not significant, but multivariate analysis indicated that it was an independent prognostic indicator for OS (HR = 4.54, $P = 0.033$)[7]. Recently, in a study by Humphris et al[47], low postoperative CA19-9 at 3 mo and before adjuvant chemotherapy were indepen-

Table 2 Clinical prognostic factors in pancreatic cancer in selected trials

Ref.	No. of patients	Results
Performance status		
Sezgin et al[25]	67	PS was an independent prognostic factor for OS
Tas et al[26]	335	Initial poor PS (2-4) was significantly associated with worse survival
DM, obesity and jaundice		
Gong et al[31]	510	HR = 1.3 for patients with BMI $\geq$ 30 compare to those with BMI < 25. But no correlation was found between BMI and survival
Yuan et al[33]	902	Higher baseline BMI was associated with reduced survival
Smith et al[34]	155	The presence of jaundice at the time of surgery was a significant adverse predictor of early survival
Strasberg et al[36]	400	The preoperative jaundice was found to be a significant indicator of poor outcome
Treatment		
Park et al[8]	340	mOS, 11.3 vs 10.4 vs 6.4 mo for stage III patients treated with CT, CCRT and BSC, respectively (P < 0.001) mOS, 6.4 vs 3.1 mo for patients with stage IV treated with CT or BSC, respectively (P < 0.001)
Lee et al[19]	82	Gemcitabine chemotherapy was found to be the only independent prognostic indicator for OS in advanced pancreatic cancer

DM: Diabetes mellitus; mOS: Median overall survival; PS:Performance status, BMI: Body mass index; CT: Chemotherapy; CCRT: Concurrent chemoradiotherapy; BSC: Best supportive care.

dent prognostic factors (median OS; 25.6 mo vs 14.8 mo, P = 0.0052) in 260 patients with pancreatic cancer who underwent surgical resection. Patients with postoperative CA19-9 levels > 90 U/mL did not benefit from adjuvant chemotherapy compared with those with a CA19-9 level of $\leq$ 90 U/mL (median OS 26.0 mo vs 16.7 mo, P = 0.0108). Normalization of CA19-9 within 6 mo of resection was also an independent favorable prognostic factor (median OS: 29.9 mo vs 14.8 mo, P = 0.0004) and normal perioperative CA19-9 levels were identified as being a good prognostic group, which was associated with a 5-year survival of 42%.

Other tumor markers

Carcinoembryonic antigen (CEA) is the standard tumor marker and is commonly used for predicting treatment response and prognosis of patients with colorectal cancer[48]. In contrast to the CA19-9 level, an impact of CEA on survival of pancreatic cancer patients has not yet been determined, but CEA might be beneficial in predicting pancreatic cancer. Zhang et al[7] in their study including 302 patients with pancreatic cancer reported that the patients with high CEA levels had a median survival of 2.0 mo compared to patients with normal levels (5.0 mo). This difference was statistically significant (HR = 1.43, P = 0.030). However, the significance of CEA levels as an independent prognostic factor could not be proved in the multivariate analysis. In a study carried out by Lee et al[22], they retrospectively analyzed 187 pancreatic cancer patients, and reported that the median OS time for patients with normal CEA levels was significantly better than that of patients with high CEA levels (16.3 mo vs 10.2 mo, P = 0.004). In addition, elevated CEA levels were found to be an independent prognostic factor in the multivariate analysis.

Despite these findings, to detect whether CEA can be applicable as a prognostic marker of pancreatic cancer, it should be evaluated in a large number of patients with all stages of pancreatic cancer. Various tumor mark-

ers such as CA125, CA15-3, CA72-4, and CA242 have also been analyzed, but their importance as independent prognostic indicators could not be definitively demonstrated[7,49].

Hematological parameters

Platelet, lymphocyte, and neutrophil counts, mean platelet volume, and the ratios of various hematologic cells have been shown to be valuable prognostic factors in various malignancies, such as renal, gynecological, and colorectal cancers[50-53]. Schwarz et al[54] demonstrated that preoperative platelet count predicts survival after resection of pancreatic adenocarcinoma. On the other hand, in a study comprising 205 patients performed by Domínguez et al[55], there was no evidence to support preoperative platelet count as either an adverse or favorable prognostic factor in pancreatic ductal adenocarcinoma, which was not compatible with a study of Zhang et al[7]. Despite conflicting results regarding platelet counts, white blood cells (WBCs) were found to be an independent prognostic factor for OS in patients with pancreatic cancer in two studies[7,46]. Although low hemoglobin levels were associated with poorer OS time, the significance as an independent prognostic marker could not be proved by the multivariate analysis[7].

The prognostic value of pretreatment platelet to lymphocyte ratio (PLR) and neutrophil to lymphocyte ratio (NLR) in patients with pancreatic cancer has also been evaluated[56,57]. Preoperative PLR has been defined as an independent significant prognostic marker by Smith et al[58] in resected pancreatic ductal adenocarcinoma. In the same study, the median overall survival in patients with a PLR of 150 or less was 19.7 mo, 13.7 mo in those with a PLR of 151-300, and 5.8 mo in patients with a value of > 300. Aliustaoglu et al[57] showed that there was no statistically significant difference between cases with PLR values $\leqslant$ 160 and > 160. However, they analyzed NLR in the same patients with pancreatic cancer. Patients with a NLR value of < 5 had a significantly higher median

Table 3 Selected trials of laboratory prognostic factors in pancreatic cancer

Ref.	No. of patients	Results
CA 19-9 levels		
Park et al[8]	340	Elevated CA19-9 levels (> 670 U/mL) were found to independent prognostic factor for OS
Zhang et al[7]	302	mOS, 3.8 mo for patients with high CA 19-9 levels vs 5.0 mo for those with normal CA 19-9 levels
Humphris et al[47]	260	mOS, 25.6 mo for low postoperative CA 19-9 levels vs 14.8 mo for high CA 19-9 levels
		Normalization of CA19-9 within 6 mo of resection was also an independent favorable prognostic factor
Other tumor markers		
Zhang et al[7]	302	mOS, 2.0 mo for patients with high CEA levels vs 5.0 mo for those with normal CEA levels
Lee et al[22]	187	mOS was 16.3 and 10.2 mo for patients with normal CEA vs high CEA levels, repsectively
Hematological factors		
Zhang et al[7]	302	WBCs were independent prognostic factor for OS
Smith et al[58]	110	mOS in patients with a preoperative PLR of 150 or less was 19.7 mo, 13.7 mo in those with a PLR of 151-300, and 5.8 mo in patients with a value of > 300
Aliustaoglu et al[57]	65	Patients with a NLR value of < 5 had a significantly higher median OS time compared to those with a NLR value of ≥ 5
Stotz et al[56]	371	An increased NLR as an independent prognostic factor for inoperable and surgically resected patients
Biochemical parameters		
Zhang et al[7]	302	Serum albumin and BUN levels were found to be independent prognostic factors for prediction of OS
Stocken et al[46]	653	Albumin, ALP, LDH, BUN, and AST were independent prognostic indicators for survival of advanced pancreatic cancer
Haas et al[60]	291	Pretreatment LDH levels were significantly associated with TTP. Baseline LDH, CRP, and bilirubin were significant prognostic factors for OS

mOS: Median overall survival; WBC: White blood cell; PLR: Platelet to lymphocyte ratio; NLR: Neutrophil to lymphocyte ratio; BUN: Blood urea nitrogen; LDH: Lactate dehydrogenase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; TTP: Time to progression; CRP: C-reactive protein; CEA: Carcinoembryonic antigen.

OS time compared to those with a NLR value of ≥ 5 ($P = 0.015$). Recently, Stotz et al[56] evaluated NLR in 371 patients with primary operable and inoperable pancreatic cancer. They reported that multivariate analysis identified increased NLR as an independent prognostic factor for inoperable PC patients (HR = 2.53, $P < 0.001$) and surgically resected pancreatic cancer patients (HR = 1.61, $P = 0.039$). Furthermore, in inoperable pancreatic cancer patients, the modified Glasgow prognostic score was associated with poor cancer-specific survival only in univariate analysis (HR = 1.44). In light of these findings, the authors concluded that risk prediction for cancer-related end points using NLR does add independent prognostic information to other well-established prognostic factors in patients with pancreatic cancer, regardless of the undergoing therapeutic modality. Thus, the NLR should be considered for future individual risk assessment in pancreatic cancer patients.

Biochemical parameters

Some serum chemistry markers such as albumin, lactate dehydrogenase (LDH), bilirubin, creatinine, and blood urea nitrogen (BUN) have previously been tested, but the prognostic role of these markers has not yet been fully defined. Serum albumin and BUN levels were found to be independent prognostic factors for prediction of survival in pancreatic cancer, while total bilirubin, direct bilirubin, glutamic-pyruvic transaminase, glutamic-oxalacetic transaminase, serum creatinine, and LDH were not[7]. However, the patients with high serum LDH levels had poor prognosis compared to those with normal levels (4.3 mo vs 7.0 mo) by univariate analysis. Tas et al[59] demonstrated that high serum LDH levels

were significantly associated with tumor burden and reflected tumor growth and invasion potential in patients with pancreatic cancer. Similarly, Stocken et al[46], in their study including 653 pancreatic cancer patients, detected that albumin, alkaline phosphatase (ALP), LDH, BUN, and aspartate aminotransferase (AST) were independent prognostic indicators for survival in patients with advanced pancreatic cancer. A recent study conducted by Haas et al[60] showed that in univariate analysis, pretreatment LDH (HR = 2.04) levels were significantly associated with time-to progression (TTP). Regarding OS, baseline LDH (HR = 2.07), C-reactive protein (CRP) (HR = 1.69), and bilirubin (HR = 1.62) were significant prognostic factors. In the multivariate analyses, pre-treatment bilirubin and CRP for OS had an independent prognostic value. They concluded that CRP, LDH, and bilirubin can also provide prognostic information on TTP and OS. Table 3 indicates selected trials of laboratory factors in pancreatic cancer.

Molecular markers

Gemcitabine is transported into the cell mainly by human equilibrative nucleoside transporter 1 (hENT1) (also known as SLC29A1). hENT1 has been investigated as a predictive biomarker of gemcitabine efficacy, mostly in pancreatic cancer, and populations of cells with lower hENT1 expression may be relatively gemcitabine resistant due to reduced intracellular accumulation of the drug[61]. Previous studies suggest that hENT1 protein expression is associated with increased OS and DFS in pancreatic cancer patients who received gemcitabine[62,63]. Recently, in patients who were included in the ESPAC 1-3 trials and were treated with adjuvant gemcitabine or

5-fluorouracil (5-FU), the results of tissue microarrays for hENT1 was presented at the 2013 ASCO annual meeting[64]. The median OS time for patients with high hENT1 expression who received gemcitabine was significantly better than that of patients with low hENT1 expression (26.2 mo *vs* 17.1 mo, $P = 0.002$). However, there was no difference among patients treated with 5-FU with respect to hENT1 expression. The authors concluded that patients with high hENT1 expression might benefit more from gemcitabine treatment.

SPARC (secreted protein and rich in cysteine), a matricellular protein found to be under-expressed in certain cancers, has emerged as a multifunctional protein capable of inhibiting the growth of pancreatic, colorectal, and ovarian cancers[65,66]. The significance of expression of SPARC as a prognostic factor in the stroma of pancreatic tumors has been shown[67]. In a study performed by Sinn *et al*[68], immunohistochemistry in the tissue sample for expression of SPARC in the stroma around the tumor, but also in the tumor cell, of patients from the Charité Onkologie (CONKO)-001 study was carried out and their results were presented at the 2013 ASCO annual meeting. Patients who received gemcitabine as adjuvant treatment had a longer DFS and OS when stromal and cytoplasmic expression of SPARC was not-strong or negative, respectively, compared with strong expression of SPARC. Thus, SPARC expression estimation, both in the tumor or its stroma, seems to be a valuable prognostic factor in patients receiving gemcitabine as adjuvant therapy in patients with pancreatic cancer.

The prognostic significance of circulating tumor cells (CTCs) has been investigated and patients who had CTCs (more than 1 in 7.5 mL) before curative surgery, or after therapy initiation, has a trend towards poorer OS or PFS[69]. Bidard *et al*[70] prospectively analyzed patients with locally advanced unresectable pancreatic cancer before and after 2 mo of chemotherapy for CTCs. More than one tumor cell in 7.5 mL was considered as positive. Before treatment, 5% of patients had positive detection of CTCs and 9% at the end of 2 mo of therapy. This positivity was found to be associated with poor tumor differentiation and the OS was shorter in these positive patients. The determination of CTCs in patients with pancreatic cancer seems to have a negative prognostic role[71]. There is a significant relationship between the amount of peritumoral CD4+ and CD8+ T-cells and survival in patients with pancreatic cancer and it was found to be an independent prognostic factor for OS[71].

Transforming growth factor β (TGF-β) acts as suppressor and promoter of cancer progression. Intracellular Smad proteins (common mediator SMAD4) play a pivotal role in mediating antimitogenic and proapoptotic effects of TGF-β[72]. In 55% of pancreatic tumors SMAD4 alterations are found and *it* is inactivated in the majority of pancreatic adenocarcinoma with concurrent mutational inactivation of the *INK4A/ARF* tumor suppressor locus and activation of the *KRAS* oncogene[73]. Previous reports revealed unclear results related with SMAD4 as

a predictor of survival in pancreatic cancer[74-76]. Blackford *et al*[76] reported that SMAD4 gene inactivation was associated with poorer prognosis in resected pancreatic adenocarcinoma. In other words, median survival time in patients without SMAD4 gene inactivation was significantly better than those with inactivation (14.2 mo *vs* 11.5 mo, $P = 0.006$). Recent study showed a significant relationship was found between SMAD4 expression and tumor size ($P = 0.006$), lymphatic invasion ($P = 0.033$), and lymph node metastasis ($P = 0.006$)[77]. Moreover, loss of SMAD4 expression was significantly associated with shorter OS and it was found to be an independent prognostic factor for both OS and DFS by multivariate analysis. Similarly, another study has confirmed these results[78].

Novel prognostic biomarkers

Hypoxia-inducible factor 1 alpha (HIF1α) has been found to be an unfavorable prognostic indicator in many cancers and is known to regulate some genes in the angiogenesis pathway[79]. Some studies have previously been showed that HIF1α had a strong impact on the prognosis of patients with pancreatic adenocarcinoma[80-82]. NEDD9, a focal adhesion scaffolding protein, has been recently proposed to regulate invasion and metastasis in some cancer types[83-85]. In a study performed by Xue *et al*[86], they investigated the expression and prognostic significance of NEDD9 in patients with pancreatic cancer. NEDD9 protein and mRNA levels were elevated in pancreatic carcinoma lesions compared with noncancerous tissues. A high NEDD9 expression level was significantly correlated with clinical staging, lymph node metastasis, and histological differentiation. The median survival time for patients with a higher NEDD9 expression was significantly shorter than that of patients with lower NEDD9 expression. In addition, the multivariate analysis revealed that NEDD9 was an independent factor of poor prognosis.

FOXM1 (Forkhead box M1) is a typical proliferation-related transcription factor and is also intimately involved in tumorigenesis. It induces cell proliferation and cell cycle progression by promoting the entry into S-phase and M-phase[87]. Xia *et al*[88] in their study, evaluated correlation between FoxM1 expression level and survival of patients with pancreatic adenocarcinoma. They showed that a high level of expression of FoxM1 was significantly correlated with clinical staging, lymph node metastasis, and histological differentiation. Furthermore, patients with a higher FoxM1 expression had a significantly shorter survival time compared to patients with lower FoxM1 expression and FoxM1 was found to be an independent factor for survival.

Recent study indicated that B7H4, HSP27 and DJ-1 protein expressions in the tissue specimens of 41 patients with resected pancreatic cancer were independently associated with a negative impact of chemotherapy with gemcitabine on patient's survival[89]. In addition, patients who overexpressed B7H4 had worse prognosis than patients without overexpression. In a study carried

Table 4 Molecular and novel biomarkers as prognostic factors in pancreatic cancer		
References	No. of patients	Results
Molecular markers		
Neoptolemos et al[64]	48	mOS, 26.2 mo for patients with high hENT1 expression *vs* 17.1 for those with low hENT1 expression who treated with gemcitabine (*P* = 0.002)
Sinn et al[68]	160	Strong stromal SPARC expression was associated with worse DFS and OS (strong *vs* not-strong DFS 9.0 *vs* 12.6 mo, *P* = 0.005; OS 19.8 *vs* 26.6 mo (*P* = 0.033).Cytoplasmic SPARC expression was also associated with worse patient outcome (positive *vs* negative DFS 7.4 *vs* 12.1 mo, *P* = 0.041; OS 14.1 *vs* 25.6 mo, *P* = 0.011) in patients with pancreatic cancer who received gemcitabine as adjuvant CT
Blackford et al[76]	114	mOS,14.2 mo in patients without SMAD4 gene inactivation *vs* 11.5 mo for those with inactivation (*P* = 0.006)
Oshima et al[77]	106	Loss of SMAD4 expression was significantly associated with shorter OS and it was found to be an independent prognostic factor for both OS and DFS
Novel biomarkers		
Xue et al[86]	106	mOS for patients with a higher NEDD9 expression was significantly shorter than that of patients with lower NEDD9 expression. NEDD9 was an independent factor of poor prognosis
Xia et al[88]	80	A higher FoxM1 expression had a significantly shorter survival time compared to patients with lower FoxM1 expression and FoxM1 was found to be an independent factor for survival

mOS: Median overall survival; DFS: Disease-free survival; hENT1: Human equilibrative nucleoside transporter 1; SPARC: Secreted protein and rich in cysteine; CT: Chemotherapy; FOXM1: Forkhead box M1.

out by Perini *et al*[90], prognostic significance of epidermal growth factor receptor (EGFR) overexpression in pancreas cancer was investigated. Univariate analysis showed that positive EGFR expression in tumor tissue had worse survival, as were male gender, portal vein resection, perineural, lymphovascular and peri-pancreatic invasion, positive margins, however, prognostic significance of positive EGFR expression as an independent prognostic factor could not be confirmed in the multivariate analysis. Selected studies associated with molecular and novel biomarkers are listed in Table 4.

CONCLUSION

The overall prognosis associated with pancreatic cancer has not improved over the last 20 years, even if new diagnostic and therapeutic strategies have emerged. So, investigations on predictive factors in pancreatic cancer are needed because these factors should have predictive value in relation to longer survival after surgery than after palliative treatment. In addition to some well-known prognostic factors such as tumor stage, surgical margin, perineural invasion, PS, treatment effect, and CA19-9, recently new prognostic indicators that have an impact on survival of patients with pancreatic cancer have appeared. The prognostic value of operative factors including OBL and PBT, NLR, and molecular markers such as SPARC, hENT1, SMAD4, CTCs, HIF1α, NEDD9 and FOXM1 has recently been shown. Prognostic factors are important and a correct definition of poor prognostic factors may help to guide more aggressive adjuvant or aggressive treatment protocols in patients with pancreatic cancer.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Targeting tight junctions during epithelial to mesenchymal transition in human pancreatic cancer

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Abstract

Pancreatic cancer continues to be a leading cause of cancer-related death worldwide and there is an urgent need to develop novel diagnostic and therapeutic strategies to reduce the mortality of patients with this disease. In pancreatic cancer, some tight junction proteins, including claudins, are abnormally regulated and therefore are promising molecular targets for diagnosis, prognosis and therapy. Claudin-4 and -18 are overexpressed in human pancreatic cancer and its precursor lesions. Claudin-4 is a high affinity receptor of Clostridium perfringens enterotoxin (CPE). The cytotoxic effects of CPE and monoclonal antibodies against claudin-4 are useful as novel therapeutic tools for pancreatic cancer. Claudin-18 could be a putative marker and therapeutic target with prognostic implications for patients with pancreatic cancer. Claudin-1, -7, tricellulin and marvelD3 are involved in epithelial to mesenchymal transition (EMT) of pancreatic cancer cells and thus might be useful as biomarkers during disease. Protein kinase C is closely related to EMT of pancreatic cancer and regulates tight junctions of normal human pancreatic duct epithelial cells and the cancer cells. This review focuses on the regulation of tight junctions *via* protein kinase C during EMT in human pancreatic cancer for the purpose of developing new diagnostic and therapeutic modalities for pancreatic cancer.

Core tip: There is an urgent need to develop novel diagnostic and therapeutic strategies to reduce the mortality of pancreatic cancer patients. In pancreatic cancer, some tight junction proteins, including claudins, are abnormally regulated and thus are promising molecular targets for Clostridium perfringens enterotoxin and monoclonal antibodies. Protein kinase C is closely related to epithelial to mesenchymal transition (EMT) of this cancer and regulates tight junctions of normal human pancreatic duct epithelial (HPDE) cells and pancreatic cancer cells. This review focuses on the regulation of tight junctions *via* protein kinase C during EMT in human pancreatic cancer compared to normal HPDE cells.

Kyuno D, Yamaguchi H, Ito T, Kono T, Kimura Y, Imamura M, Konno T, Hirata K, Sawada N, Kojima T. Targeting tight junctions during epithelial to mesenchymal transition in human pancreatic

INTRODUCTION

Pancreatic cancer continues to be a leading cause of cancer-related death worldwide due to late detection, lack of therapeutic targets and ineffective therapies. At the time of diagnosis, few patients with pancreatic cancer present with localized disease amenable to surgical resection, while the remaining patients present with locally advanced or distant metastasis. It exhibits the poorest prognosis of all solid tumors with a 5-year survival rate < 5% and a median survival of 3-6 mo after diagnosis[1]. Thus, there is an urgent need to develop novel diagnostic and therapeutic strategies to reduce the mortality of these patients.

Transition of a cancer cell from an epithelial to mesenchymal morphology leads to increased migratory and invasive properties, and thus facilitates the initiation of metastasis in pancreatic cancer[2,3]. The epithelial to mesenchymal transition (EMT) is characterized by a loss of cell-cell contact and apicobasal polarity. The hallmarks of EMT *in vitro* and *in vivo* include the upregulation of mesenchymal markers, the downregulation of epithelial cell adhesion molecules including tight junction proteins, and dysfunction of the tight junction fence[4,5]. EMT is accompanied by loss of occludin and claudins as well as E-cadherin *via* the Snail family[6-9]. The transcription factor Snail, which has high to moderate expression in 78% of pancreatic ductal adenocarcinoma specimens, appears to promote metastasis and chemoresistance in pancreatic cancer[10,11]. The activation of protein kinase C (PKC) is known to be involved in EMT in various type of cancer including pancreatic cancer. The PKC activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induces EMT in human prostate cancer cells[12] and pancreatic cancer cell line HPAC[13]. Expression of PKCα and PKCδ closely contributes to EMT in colon cancer cells[14,15]. Transforming growth factor-β1 (TGF-β1), which promotes EMT in pancreatic cancer cells[16], induces PKCα in poorly differentiated pancreatic cancer cell line BXPC-3[17].

In several human cancers, including pancreatic cancer, some tight junction proteins are abnormally regulated and therefore promising molecular targets for diagnosis and therapy[18,19]. The current review will focus on the roles of tight junction proteins, including claudins, and PKC signaling with regard to the potential applicability for diagnosis, prognosis and the therapy during EMT in pancreatic cancer.

TIGHT JUNCTION AND ITS PROTEINS

Epithelial cells including pancreatic epithelial cells are bordered by two functionally and biochemically different membranes[20]. This integrity is maintained by intercellular junctional complexes, such as tight junctions, adherent junctions, and desmosomes[21]. Tight junctions are the most apical components of intercellular junctional complexes in epithelial and endothelial cells. They separate the apical and basolateral cell surface domains, maintaining cell polarity (termed the "fence" function), and selectively control solute and water flow through the paracellular space (termed the "barrier" function)[22-25]. They also participate in signal transduction mechanisms that regulate epithelial cell proliferation, gene expression, differentiation and morphogenesis[26]. The tight junction is formed by integral membrane proteins and peripheral membrane proteins. The integral membrane proteins are claudins[27,28], occludin[29], tricellulin[30], marvelD3[31] and junctional adhesion molecules[32] (Figure 1). Peripheral membrane proteins include the scaffold PDZ-expression proteins zonula occludens (ZO)-1, ZO-2, ZO-3, multi-PDZ domain protein-1, membrane-associated guanylate kinase with inverted orientation-1 (MAGI)-1, MAGI-2, MAGI-3, cell polarity molecules atypical PKC isotype-specific interacting protein/PAR-3, PAR-6, PALS-1, and PALS-1-associated tight junction, as well as the non-PDZ-expressing proteins cingulin, symplekin, ZONAB, GEF-H1, aPKC, PP2A, Rab3b, Rab13, PTEN, and 7H6[21,33,34]. These tight junction proteins are regulated by various cytokines and growth factors *via* distinct signal transduction pathways including PKC[35,36].

The claudin family, which consists of at least 27 members, is solely responsible for forming tight junction strands and has four transmembrane domains and two extracellular loops[21,37] (Figure 2). The first extracellular loop is the coreceptor of hepatitis C virus[38] and influences the paracellular charge selectivity[39], and the second extracellular loop is the receptor of Clostridium perfringens enterotoxin (CPE)[40].

Both occludin and tricellulin (marvelD2) contain the tetra-spanning MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain that is present in proteins involved in membrane apposition and concentrated in cholesterol-rich microdomains[41]. The novel tight junction protein marvelD3 contains a conserved MARVEL domain like occludin and tricellulin[31,42].

In general, cancer cells lose their specific functions and polarity with a decrease in the development of tight junctions. It is thought that the loss of tight junction functions in part leads to invasion and metastasis of cancer cells[43].

Tight junction proteins are dysregulated during carcinogenesis and EMT. Expression of some claudin family members is significantly altered by epigenetic regulation in human cancer[44-46].

EXPRESSION PATTERNS AND THE ROLE OF TIGHT JUNCTION PROTEINS IN NORMAL PANCREAS

Several tight junction proteins are expressed in a tissue-specific and organ-specific manner[47-49]. Normal ductal
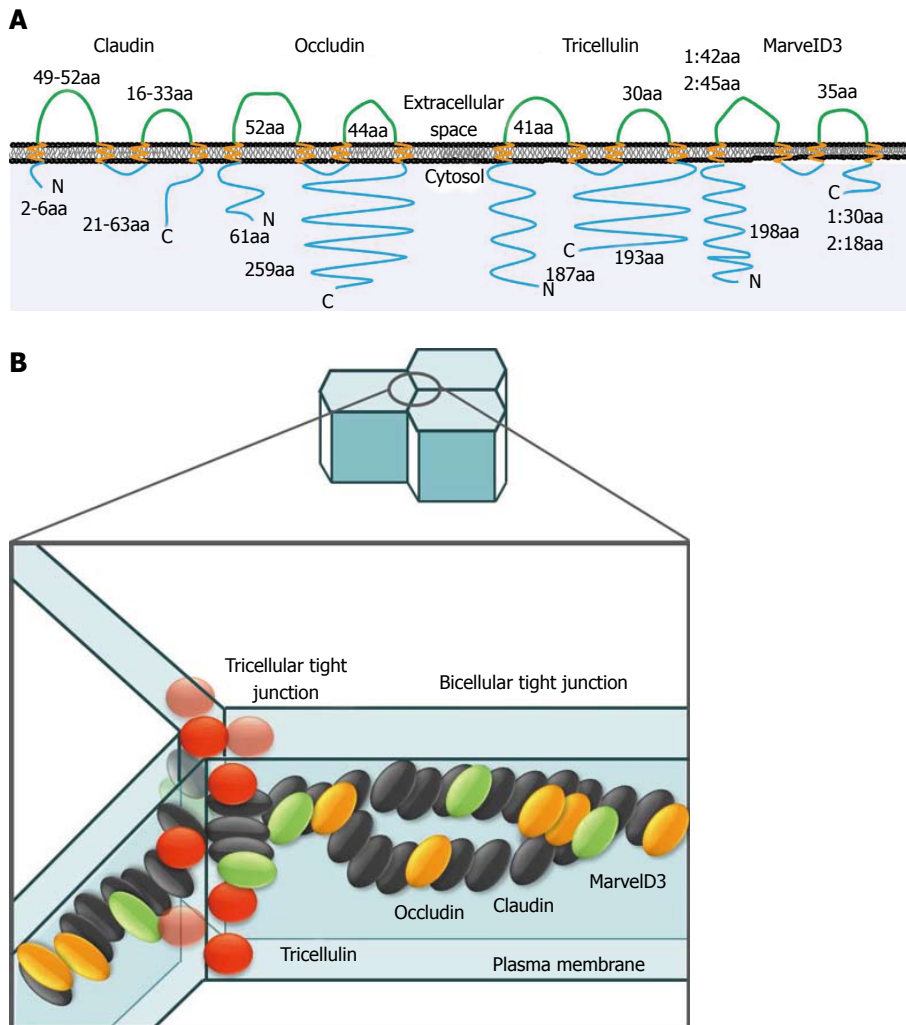
Figure 1 Claudins, occludin, tricellulin, marvelD3 and junctional adhesion molecules. A: Schematic representation of human claudin, occludin, tricellulin, and marvelD3. These molecules contain four transmembrane domains with two extracellular loops. Claudins consist of at least 27 members. Occludin has several variants. MarvelD3 has two isoforms. aa: amino acid; B: Models of tight junction protein locations in paracellular space. The bicellular tight junction is the interface between two cells, whereas the vertex where three cells meet is termed the tricellular tight junction. The tight junction strands within both bicellular and tricellular regions are composed of claudins (black ellipses). MarvelD3 (green ellipses), occludin (orange ellipses), and tricellulin (red spheres) incorporated into claudin-based tight junction strands. Occludin and tricellulin are primarily found at bicellular and tricellular regions, respectively, whereas marvelD3 is present at both sites. Tricellulin is unique in that it is present at the tight junction and along the lateral membrane.

and acinar structures of the pancreas express claudin-1, -2, -3, -4, and -7, whereas endocrine cells within the islets of Langerhans express claudin-3 and -7 (Figure 3)[50,51]. Pancreatic duct cells deliver the enzymes produced by acinar cells into duodenum and secrete a HCO_3^--rich fluid to neutralize gastric acid from the stomach[52]. Tight junctions of the pancreatic duct form the pancreatic ductal barrier. Freeze-fracture analysis of the pancreatic duct reveals that tight junctions contained a parallel array of three to five continuous sealing strands and the pancreatic enzymes cannot leak out from the lumen into the intercellular spaces (Figure 3)[53,54]. Tight junctions of the pancreatic duct are also regulators of physiologic secretion of the pancreas. Pancreatic ductal tight junctions, which is leaky and has the function of selective permeability, may play a role of channels of Na^+ and HCO_3^- *via* paracellular pathway[55,56].

The tight junctions of pancreatic duct epithelial cells and exocrine cells are dynamic structures that can be disrupted by various external stimuli including ductal hypertension[57,58]. The disruption of pancreatic duct tight junctions is an early event in different types of pancreatitis[59-64]. Although dysfunction of tight junctions in pancreatic duct is observed by various pathological conditions, the regulatory mechanisms of tight junctions remain unknown even in normal human pancreatic duct epithelial (HPDE) cells.

EXPRESSION PATTERNS OF TIGHT JUNCTION PROTEINS IN PANCREATIC CANCER

The tight junction protein expression pattern varies be-
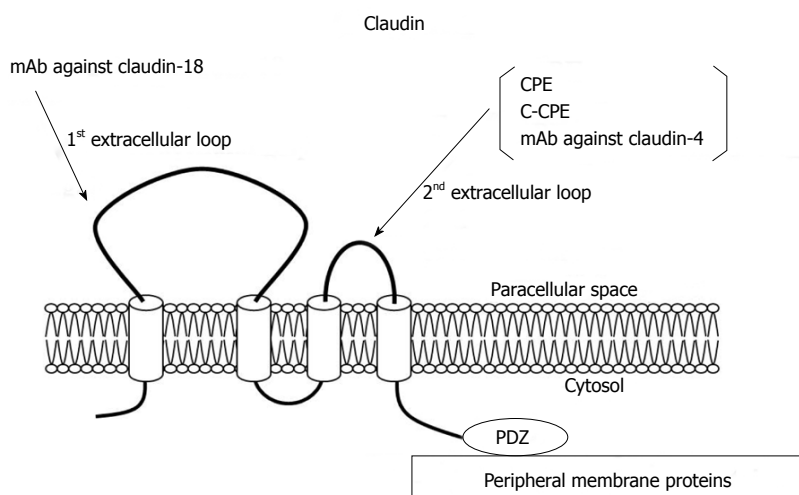
Claudin

Figure 2 Structures of claudins. The first extracellular loop of claudin-18 targeted for therapy using monoclonal antibodies and the second extracellular loop of claudin-4 targeted for therapy using monoclonal antibodies, Clostridium perfringens enterotoxin and C-Clostridium perfringens enterotoxin.
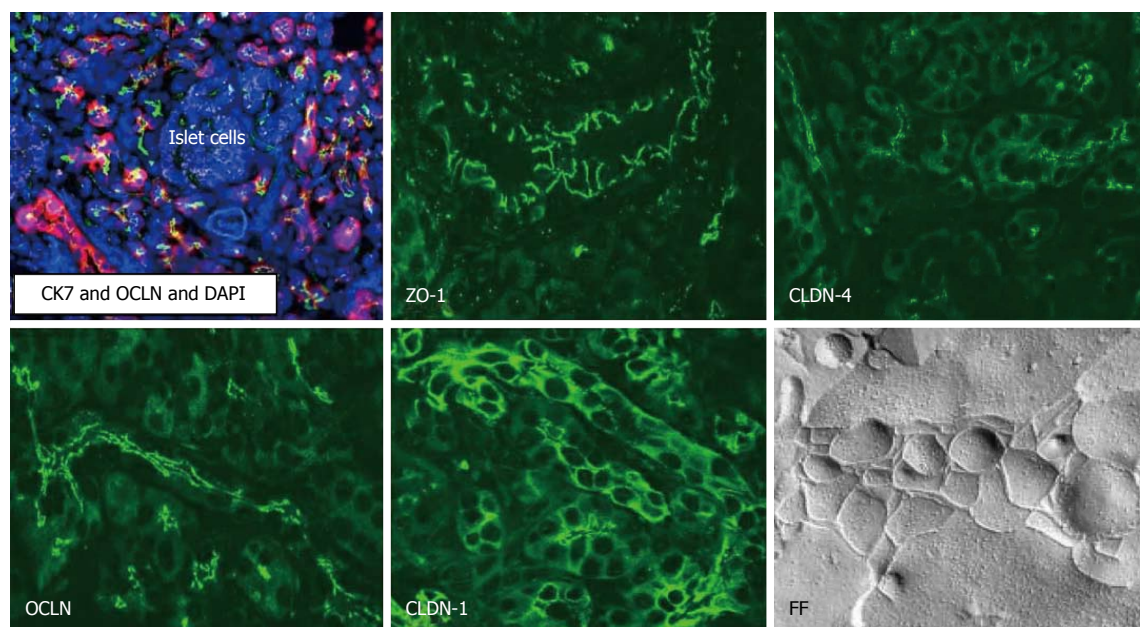
Figure 3 Localization and structures of tight junctions in normal human pancreas. In normal pancreatic ducts which express CK (Cytokeratin)7, occludin (OCLN), ZO-1 and claudin (CLDN)-1, -4 are observed by immunostaining. In freeze-fracture (FF) replica, well-developed tight junction strands are observed in normal pancreas.

tween normal pancreatic tissue and pancreatic cancer. Claudin-1, -4, -7 and -18 are positive in pancreatic adenocarcinoma, whereas endocrine tumors are negative for claudin-1 and -4. Claudin-3 and -7 proteins are detected in endocrine tumors, whereas claudin-13 is negative in ductal adenocarcinoma[18,50,51]. Claudin-1, -2 and -4 are detected in exocrine tumors[65]. In borderline cystic tumors the level of claudin-1, -4 and -7 protein expression is between that of benign and malignant tumors[65]. This supports the sequential development theory regarding mucinous cystic tumors.

Liver metastasis of pancreatic cancer is strongly positive for claudin-4, weakly positive for claudin-1, and negative or faintly positive for claudin-7[66]. It is interesting

that claudin-3 is positive in liver metastasis of pancreatic cancer whereas claudin-3 staining is not detected in primary pancreatic cancer[50,66].

A study investigating ZO-1 in pancreatic cancer showed that expression of ZO-1 was increased in pancreatic adenocarcinoma samples in comparison with normal samples[67]. In pancreatic cancer cells, ZO-1 protein translocalizes from apical and apicolateral areas to the cytoplasm and nucleus, and translocation of ZO-1 is involved in the induction of invasion through epidermal growth factor receptor (EGFR) activation[68].

We established human telomerase reverse transcriptase-transfected HPDE cells as models of normal pancreatic duct epithelial cells[51]. The hTERT-HPDE cells

are positive for HPDE cell markers such as CK7, CK19 and carbonic anhydrase isozyme 2 and express epithelial tight junction molecules claudin-1, -4, -7 and -18, occludin, tricellulin, marvelD3, JAM-A, ZO-1, and ZO-2[51]. The expression patterns of tight junction molecules in the hTERT-HPDE cells are similar to those of pancreatic tissues *in vivo*[51].

CLAUDIN-1 IN NORMAL PANCREATIC DUCT AND CANCER

Claudin-1 is expressed in various types of epithelial cells, and plays an important role in epithelial cell polarity and cancer invasion and metastasis[69-72]. However, its role remains controversial far in various cancers. In pancreatic cancer, claudin-1 expression is responsible for tumor necrosis factor α-dependent cell growth signals that lead to apoptosis and the inhibition of cell proliferation[73]. Claudin-1 is localized at the cell membranes of normal pancreatic ducts and well-differentiated pancreatic carcinoma, whereas in poorly differentiated pancreatic carcinoma it is weakly detected in cytoplasm[74].

EMT is associated with the simultaneous repression of the genes encoding E-cadherin, claudins and occludin[8]. The transcription factors Snail and Slug, which play a central role in EMT, bind to the E-box motifs present in the claudin-1 promoter and have a critical negative regulatory role in malignant cancer cell lines that express low levels of the claudin-1 transcript[8,75]. Treatment with TGF-β1 induces EMT in pancreatic cancer cells and TGF-β upregulates Snail and downregulates claudin-1, -4 and occludin in PANC-1 cells[74]. Taken together, this indicates that claudin-1 may be a potential biomarker for the development of pancreatic cancer. Thus further investigation of the significance of claudin-1 in pancreatic cancer cells and normal pancreatic duct epithelial cells is required.

CLAUDIN-4 IN NORMAL PANCREATIC DUCT AND CANCER

DNA microarray, immunohistochemical, and quantitative real-time reverse transcription-polymerase chain reaction analyses have provided evidence that claudin-4 is upregulated in pancreatic cancer tissues[76]. Furthermore, claudin-4 is also overexpressed in pancreatic intraepithelial neoplasia (PanIN), intraductal papillary neoplasia (IPMN), and mucinous cystic neoplasia (MCN), and is correlated with the histological tumor grade in both IPMN and MCN[77,78]. On the other hand, overexpression of claudin-4 decreases the invasiveness and metastatic potential of pancreatic cancer cells *in vitro*[19]. Patients with high expression of claudin-4 mRNA and protein survive longer than those with low claudin-4 expression[79].

Claudin-4 is also a high-affinity receptor of CPE[80]. The 35-kDa polypeptide CPE causes food poisoning in humans, binds to its claudin receptor, and then causes changes in membrane permeability *via* formation of a complex on the plasma membrane followed by the induction of apoptosis[81]. Full-length CPE with a direct cytotoxic effect and the COOH-terminal receptor-binding domain of CPE (C-CPE) without a cytotoxic effect are employed as selective treatment and drug delivery systems against claudin-4 expressing pancreatic tumors[82,83].

CPE induces an acute dose-dependent cytotoxic effect in claudin-4-expressing nude mouse xenografts of PANC-1, which is a poorly differentiated pancreatic cancer cell line[82,84]. In the pancreatic cell lines PANC-1, BXPC-3, HPAF-Ⅱ and HPAC, claudin-4 is found not only at the apicalmost regions but also at basolateral membranes[85]. When these pancreatic cancer cell lines are treated with CPE, it induces dose-dependent cytotoxic effects in all of them[85]. Furthermore, in HPAC cells, the cytotoxicity of CPE is significantly decreased by knockdown of claudin-4 by siRNAs[85].

In hTERT-HPDE cells cultured with 10% FBS, claudin-4 is localized at the apicalmost regions, which are tight junction areas[85]. When hTERT-HPDE cells cultured with 10% FBS in which the expression of claudin-4 protein is as high as in pancreatic cell lines in Western blotting, are treated with CPE, cytotoxicity is not observed even at high concentrations of CPE[85]. These findings suggest that, in pancreatic cancer cells, CPE binds to the free second extracellular loop of claudin-4 outside of tight junctions and that, in normal HPDE cells, it cannot bind to that of claudin-4 in tight junction areas.

EFFECT OF C-CPE TARGETING CLAUDIN-4 AGAINST PANCREATIC CANCER

The functional domains of CPE can be separated into a receptor-binding region (C-terminal of CPE, C-CPE) and cytotoxic region (N-terminal of CPE). C-CPE is a C-terminal fragment composed of the CPE amino acids 184 to 319[80]. The receptor binding region of CPE has been reported to be in the C-terminal 30 residues (amino acids 290 to 319) of CPE[86].

C-CPE is a nontoxic molecule that disrupts the tight junction barrier function and enhances cellular absorption[87]. It enhances the effectiveness of clinically relevant anticancer agents such as Taxol and carboplatin against cancer cells[88]. In our study, when HPAC cells were treated with C-CPE, the barrier function was markedly decreased at a nontoxic concentration of C-CPE and recovered in the absence of C-CPE (personal data). C-CPE may enhance the effectiveness of clinically relevant chemotherapies in pancreatic cancer.

The development of molecular imaging approaches using tissue- and cell-specific tracers plays a crucial role to improve early diagnosis and therapy in cancer. Claudin-4 is utilized as a target for imaging of pancreatic cancer. Non-cadmium-based quantum dots bioconjugated to claudin-4 monoclonal antibodies are used as highly ef-

ficient, nontoxic optical probes for imaging live pancreatic cancer cells *in vivo* and *in vitro*[89]. C-CPE labelled with a cyanine dye with novel optical imaging methods, 2D planar fluorescence reflectance imaging technology and 3D fluorescence-mediated tomography, enables noninvasive visualization of claudin-4 positive pancreatic cancer and its precursor lesions[90]. Furthermore, it is thought that C-CPE can be used as a carrier for other bacterial toxins to claudin-4-positive cancer cells. A claudin-4-targeting antitumor molecule that consisted of C-CPE fused to protein synthesis inhibitory factor derived from *Pseudomonas aeruginosa* exotoxin or diphtheria toxin fragment A (DTA) were especially toxic to claudin-4 positive cancer cells in vivo and *in vitro*[83,91,92].

CLAUDIN-7 IN NORMAL PANCREATIC DUCT AND CANCER

Claudin-7 is expressed in various types of epithelial cells and directly interacts with EpCAM, forming a complex with CD44 variant isoforms and tetraspanins outside of tight junction areas[93,94]. Furthermore, EpCAM is one of the surface markers in pancreatic cancer stem cells[95], and claudin-7 regulates the EpCAM-mediated functions in tumor progression such as proliferation, migration, and anti-apoptosis[96,97]. Claudin-7 supports tumorigenic features of EpCAM by provoking EpCAM cleavage and its cotranscription factor activity, and is directly engaged in motility and resistance to apoptosis in rat pancreatic cancer[98].

In human pancreatic ductal adenocarcinoma, there is a gradual decline in membrane-bound expression of claudin-7 immunoreactivity in parallel with the degree of tumor differentiation[99]. Claudin-7 expression also appears to be inversely associated with the gland size in tumors, with large neoplastic glands displaying more frequent claudin-7 positivity than smaller glands[99]. There is no association between claudin-7 and tumor size, the presence of nodal metastases or survival of the patients, indicating that while expression of claudin-7 is related to differentiation of ductal pancreatic adenocarcinoma it does not influence tumor progression[99].

In a human pancreatic cancer cell line and hTERT-HPDE cells, ELF3 is associated with claudin-7[51]. ELF3 belongs to the ELF (E74-like factor) subfamily of the ETS transcription factors, but it is distinguished from most ETS family members by its expression pattern, which is specific in epithelial tissues of the lung, liver, kidney, pancreas, prostate, small intestine, and colon mucosa[100]. ELF3 controls intestinal epithelial differentiation[101]. It is reported that the expression of claudin-7 in epithelial structures in synovial sarcoma is regulated by ELF3[102]. Thus, the expression of claudin-7 and its regulation *via* ELF3 may be important as potential therapeutic targets for pancreatic cancer.

CLAUDIN-18 IN NORMAL PANCREATIC DUCT AND CANCER

In pancreatic cancer, claudin-18 is as highly expressed as claudin-4[18]. Claudin-18 has two alternatively spliced variants, claudin-18a1 and claudin-18a2, which are highly expressed in the lung and stomach, respectively[103]. Claudin-18a2 is activated in a wide range of human malignant tumors, including gastric, esophageal, pancreatic, lung, and ovarian cancers, and can be specifically targeted by monoclonal antibodies against the first extracellular loop[44]. Claudin-18 is highly expressed in PanIN, IPMN, MCN, pancreatic duct carcinoma, and metastases of pancreatic cancer, and serves as a diagnostic marker[18,78,99,104-106]. Neuroendocrine neoplasia is found positive with low rates[105]. Thus, claudin-18 could be useful as a putative marker and therapeutic target for neoplasia of the pancreas. Furthermore, because claudin-18 expression is most pronounced in well-differentiated pancreatic cancers, and patients with high expression of claudin-18 survive longer than those with low claudin-18 expression[18], its expression level may also have prognostic implications for patients with pancreatic cancer.

TRICELLULIN IN NORMAL PANCREATIC DUCT AND CANCER

Tricellulin was identified as the first marker of the tricellular tight junction, which formed at the meeting points of three cells[30]. It is required for the maintenance of the transepithelial barrier and expressed in both the normal pancreatic duct and pancreatic cancer[30,107,108]. It is one of three members of the tight junction-associated MARVEL protein family. The other two members are occludin and marvelD3[31,42]. Occludin and tricellulin are present at bicellular and tricellular tight junctions, respectively, whereas marvelD3 is present at both sites[31,42]. Both normal and neoplastic pancreatic exocrine tissues express tricellulin, whereas no expression is seen in normal or neoplastic endocrine cells[108]. Tricellulin expression in pancreatic ductal adenocarcinomas shows a significant negative correlation with the degree of differentiation[108].

Tricellulin expression in tricellular tight junctions is strongly regulated together with the barrier function *via* the c-Jun N-terminal kinase (JNK) transduction pathway[109]. Activation of JNK promotes the development of various tumors[110-112]. Furthermore, JNK inhibitors decrease the growth of human and murine pancreatic cancers *in vitro* and *in vivo*[113]. Tricellulin expression and the barrier function are upregulated together with the activity of phospho-JNK by treatment with the JNK activator anisomycin in HPAC cells[109]. In hTERT-HPDE cells, tricellulin expression is significantly increased by all JNK activators, similar to the response in HPAC cells[109].

JNK may be involved in the regulation of tight junctions, including tricellulin expression and the barrier function in normal pancreatic duct epithelial cells, and may be a potential therapeutic target for pancreatic cancer.

MARVELD3 IN NORMAL PANCREATIC DUCT AND CANCER

MarvelD3, the novel tight junction protein, is transcriptionally downregulated in poorly differentiated pancreatic cancer cells, whereas it is maintained in well-differentiated human pancreatic cancer cells and normal pancreatic duct epithelial cells[114]. Furthermore, marvelD3 is transcriptionally downregulated in Snail-induced EMT during the progression of pancreatic cancer[114]. Therefore, marvelD3 could be a new marker during pancreatic cancer progression. However, little is known about the detailed role of marvelD3 in epithelial tight junctions and how it is regulated in various types of cells, including normal pancreatic duct epithelial cells and pancreatic cancer cells.

ROLE OF PKC IN TIGHT JUNCTIONS DURING EMT IN NORMAL PANCREATIC DUCT AND CANCER

PKC belongs to the family of serine-threonine kinases and regulates various cellular functions[115]. It has been shown to induce both assembly and disassembly of tight junctions depending on the cell type and conditions of activation[116-118]. At least 12 different isozymes of PKC are known and can be subdivided into three classes (classic or conventional, novel and atypical isozymes) according to their responsiveness to activators[119,120]. The levels of PKCα, PKCβ1, PKCδ and PKCι are higher in pancreatic cancer, whereas that of PKCε is higher in normal tissue[121,122]. In pancreatic cancer, tumorigenicity is directly related to PKCα expression, as demonstrated by decreased survival when it is overexpressed[123]. The increased level of PKCα is also associated with pancreatic cancer cell proliferation[124].

Tight junction proteins are regulated by various cytokines and growth factors *via* distinct signal transduction pathways including PKC[35,36]. In various cancer cells, the regulation of tight junctions *via* PKC pathway is reported. The assembly of ZO-1 and occludin is involved in PKC-dependent signaling in gastric cancer cells[125]. The activation of c-Abl-PKCδ signaling pathway is critically required for the claudin-1-induced acquisition of the malignant phenotype in human liver cells[72]. PKC activation causes an increase in claudin-1 transcription and claudin-1 appears to contribute to cell invasion in human melanoma cells[126]. PKCε activation regulates an α5 integrin-ZO-1 complex and correlates with invasion and unfavorable prognosis in lung cancer cells[127].

We have previously reported that the regulation of tight junctions in normal human pancreatic duct epithelial cells and pancreatic cancer cells is closely associated with PKC and PKC-induced transcriptional factors[13,51,74,104,109,128]. To confirm whether the PKC signal pathway was closely associated with the regulation of tight junctions, hTERT-HPDE cells and pancreatic cancer cells were treated with the PKC activator TPA and the specific PKC isoform inhibitors. Treatment with TPA enhanced expression of claudin-1, -4, -7, and -18, occludin, JAM-A and ZO-1, -2[51]. The upregulation of claudin-4 by TPA was prevented by a PKCα inhibitor and the upregulation of claudin-7, occludin, ZO-1 and ZO-2 was prevented by a PKCδ inhibitor[51]. In HPAC cells, tricellulin was in part regulated *via* PKCδ and PKCε pathways[109], and the expression of claudin-18 and localization of claudin-4 and occludin were in part regulated *via* a PKCα pathway[13,104,128]. Claudin-18 mRNA and protein, indicated to be claudin-18a2, were markedly induced by TPA in well- and moderately differentiated human pancreatic cancer cell lines HPAF-Ⅱ and HPAC and hTERT-HPDE cells[104]. The upregulation of claudin-18 by TPA in human pancreatic cancer cell lines was prevented by inhibitors of PKCδ, PKCα and PKCε, whereas the upregulation of claudin-18 by TPA in hTERT-HPDE cells was prevented by inhibitors of PKCδ, PKCα and PKCθ[104].

On the other hand, a PKCα inhibitor enhances sensitivity of HPAC cells to CPE by preventing mislocalization of claudin-4[13], and prevents downregulation of claudin-1 during EMT of pancreatic cancer cells[74]. The TGF-β-PKCα-PTEN cascade is a key pathway for pancreatic cancer cells to proliferate and metastasize[129]. The PKC may be a useful target for pancreatic cancer therapy[119] and PKCα inhibitors may be potential therapeutic agents against the malignancy of human pancreatic cancer cells[130]. Further study of the tight junctions of normal HPDE cells and pancreatic cancer cells *via* PKC pathways including isoforms is important for not only physiological regulation of tight junction molecules but also for therapeutic targeting of pancreatic cancer cells. In addition to PKC pathway, other signaling pathways including Ras/ERK1/2, Smad/STAT3, Notch, Wnt and Src are closely related to EMT of pancreatic cancer[131-135]. However, the regulation of tight junctions in normal pancreatic duct and pancreatic cancer *via* these signal pathways remain unknown.

CONCLUSION

The signaling pathways including PKC regulate tight junctions during EMT in pancreatic cancer. By using hTERT-HPDE cells, we found that the expression of tight junction proteins in normal HPDE cells was regulated by various factors. For developing new diagnostic and therapeutic modalities *via* tight junction molecules in pancreatic cancer, it is necessary to investigate the profile and the regulation of tight junctions in normal HPDE cells as well as pancreatic cancer cells.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Novel therapeutic targets for pancreatic cancer

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Abstract

Pancreatic cancer has become the fourth leading cause of cancer death in the last two decades. Only 3%-15% of patients diagnosed with pancreatic cancer had 5 year survival rate. Drug resistance, high metastasis, poor prognosis and tumour relapse contributed to the malignancies and difficulties in treating pancreatic cancer. The current standard chemotherapy for pancreatic cancer is gemcitabine, however its efficacy is far from satisfactory, one of the reasons is due to the complex tumour microenvironment which decreases effective drug delivery to target cancer cell. Studies of the molecular pathology of pancreatic cancer have revealed that activation of KRAS, overexpression of cyclooxygenase-2, inactivation of p16^{INK4A} and loss of p53 activities occurred in pancreatic cancer. Co-administration of gemcitabine and targeting the molecular pathological events happened in pancreatic cancer has brought an enhanced therapeutic effectiveness of gemcitabine. Therefore, studies looking for novel targets in hindering pancreatic tumour growth are emerging rapidly. In order to give a better understanding of the current findings and to seek the direction in future pancreatic cancer research; in this review we will focus on targets suppressing tumour metastatsis and progression, KRAS activated downstream effectors, the relationship of Notch signaling and Nodal/Activin signaling with pancreatic cancer cells, the current findings of non-coding RNAs in inhibiting pancreatic cancer cell proliferation, brief discussion in transcription remodeling by epigenetic modifiers (*e.g.*, HDAC, BMI1, EZH2) and the plausible therapeutic applications of cancer stem cell and hyaluronan in tumour environment.

Core tip: Some of the targets discussed here have been discovered to enhance the effectiveness of gemcitabine upon co-administration of the corresponding agents, for instance, hyaluronidase can deplete hyaluronan in stromal region to enhance gemcitabine delivery. Besides, some signaling molecules, *e.g.*, RalGEF-RAl, Rac1, and PAR2 are being targeted to suppress metastasis. Tumour proliferation is limited upon DR5 activated apoptosis and others promising therapeutic areas like epigenetic modifiers; IAP, miR, lncRNA, and cancer stem cells-tumour microenvironment will also be discussed.

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer death in the last two decades because of various obsta-

cles in its treatment[1]. Late and poor prognosis are two of the causes of high fatality rate[2]. Patients diagnosed having pancreatic cancer are usually at their very late stage and spreading of the highly metastatic pancreatic cancer cell into the lymphatic system and vicinal organs limited the choices of effective treatments[3].

Gemcitabine is the current standard chemotherapy for pancreatic cancer[4], however, due to the complex tumour microenvironment[5] and high metastatic property of pancreatic cancer. The effectiveness of gemcitabine in treating pancreatic cancer is unsatisfactory. Studies of targeting the molecular pathology have been carried out to quest for more potential targets; for instance, activation of oncoprotein V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS)[6], overexpression of cyclo-oxygenase-2[1], inactivation of p16[INK4A][6] and loss of p53 activities[6] mark the onset of pancreatic cancer. Because, the treatments of targeting these molecules can enhance the efficacy of gemcitabine in pancreatic cancer[3], these imply that combinatorial therapies may be the future direction in treating pancreatic cancer[7]. Therefore, in the following context of this review we are going to briefly evaluate plausible therapeutic targets, in terms of the molecular and cellular level which covers the roles of several signal transducers, signaling pathways, surface proteins, receptor proteins, non-coding RNA, epigenetic modifiers and tumour microenvironment in driving pancreatic cancer and to explore any possibilities of combinatorial therapy among them.

SIGNAL TRANSDUCERS

CTHRC1

Collagen Triple Helix Repeat Containing-1 (CTHRC1) is a secretory protein[8], which participates in vascular remodulation through limiting collagen matrix deposition[9], and also morphogenesis but most importantly enhancing cell migratory ability and adhesiveness in tumour cells[8]. CTHRC1 is found expressed in a wide spectrum of human cancer cells, and is in particular found highly expressed in pancreatic cells[10]. The CTHRC1 protein is found highly expressed in invasive melanoma but weakly expressed or absent in benign nevi or non-invasive melanoma[8]. Over expression of CTHRC1 in pancreatic cancer has enhanced the tumour cells migration and metastatic properties; studies of using induced hyper CTHRC1 expressed pancreatic cancer cell, MiaPaCa-2-CTHRC1 and shRNA-CTHRC1 suppressed pancreatic cancer cells, BxPC3 and Panc1, are used to evaluate CTHRC1 on pancreatic cancer cells metastatic in *in vivo* mice model[10]. The result has revealed a wider metastatic spread of hyper CTHRC1 expressed pancreatic cancer cell to secondary organs while the hypo CTHRC1 expressed pancreatic tumour cells has reduced tumour cells spreading to neighboring organs when compared with the tumour cells transfected with control shRNA[10]. The phosphorylation of Focal adhesion kinase (FAK)-steroid receptor coactivator (Src) cascade and extracel-

lular signal-regulated kinases (Erk) are the causes of the enhanced metastasis[10], it is found that the binding of CTHRC1 onto the wingless-type MMTV integration site family protein, member 5A (Wnt5a) can stabilize the Wnt receptor complex[11] and the facilitated binding of the Wnt5a into its Wnt receptor complex will activate paxillin which leads to phosphorylation of Src-FAK signaling cascade and Erk[10], as both Src and Erk signaling pathways could lead to tumour progression and enhanced motility[12], overexpression of CTHRC1 has increased the phosphorylation of Src and Erk, and vice versa[10], these indicating the CTHRC1 plays a critical role in controlling pancreatic tumour cell adhesiveness and metastasis. Besides, activating the fore mentioned kinases, CTHRC1 is reported to repress the production of collagen I into the stromal environment of pancreatic cancer[8], supporting of its role as a cancer metastasis enhancing gene.

As suppressing CTHRC1 can reduce the metastatic and motility of pancreatic cancer cell, future studies can investigate on the feasibility of combining CTHRC1 targeted therapy with current anti pancreatic cancer drugs. CTHRC1 appears as a promising target in sequestering pancreatic cancer from spreading to neighboring organs, however, whether it could sensitize the tumour cells to current anti-cancer treatments in pancreatic cancer is not yet published. CTHRC1 would be a more promising target if it is proved to sequester pancreatic cancer during chemotherapy, providing a higher chance in elimination of tumour cells in the patient.

RAC1

RAS-related C3 botulinum toxin substrate 1 (Rac1)[13] is found to be an important factor in regulating pancreatic islet morphogenesis[14], failure of cell spreading has been reported on gelatin-coated culture by blocking Rac1 in isolated islet cells[14]. Apart from its vital role in directing organogenesis, Rac1 is one of the Rat sarcoma (Ras) effectors[15] and is being overexpressed in pancreatic cancer[16]. It has been found diminishing the formation of acinar-ductal metaplasia (ADM), pancreatic intraepithelial neoplasia (PanIN) and tumours when its expression is ablated in K-Ras[G12D] induced pancreatic ductual adenocarcinoma (PDAC) mice model[15]. In cancer biology, Rac1 is found to promote tumour migration and metastasis through lamellipodia production[17]. Studies of targeting Rac1 may be beneficial in slowing down the spreading of pancreatic cancer cells.

Two guanine nucleotide exchange factors (GEFs) have been reported activating Rac1, dynamin 2 (Dyn2) has been reported regulating Rac1 in an undefined mechanism[15]; Dyn2 is found associated with vav 1 guanine nucleotide exchange factor (Vav1) in coimmunopreciptation, an onco-protein acts as a guanine nucleotide exchange factor (GEF) in Rac1 activation, and Vav1 is stabilized by the degradation of lyzozyme and heat shock cognate 70 upon binding with Dyn2[15]. Truncated form of Dyn2 has found unable to associate with Vav1 and

leading to reduced activation of Rac1 by 50%[15]. However, cell lines deficit in Vav1 expression (*e.g.*, Panc1) would undermine this therapeutic direction[15]. Another GEF, T lymphoma invasion and metastasis 1 (Tiam1), which is reported as an oncogene and associated with various cancers, Tiam1 directs Rac1 to enhance tumour proliferation and metastasis through the Wnt signaling pathway, however, suppressions of Tiam1 and Rac1 will lead to the activation of another oncoprotein, RhoA, which also promotes pancreatic tumour cells aggressiveness and metastasis[17]. The tumour growth and long term survival are significantly suppressed and enhanced respectively, upon simultaneous inhibition of Rac1 and RhoA[17]. These revealed the invasiveness and tumour migration of pancreatic tumour cells are under complex controls, balanced Rac1 and RhoA expression level is suggested to be one of those[17], and the possibility of the participation of Dyn2 in between Rac1 and RhoA, as the effect of reduction of activated Rac1 in truncated Dyn2 experiment on RhoA is unknown.

However, when shifting to microRNA research, microRNA-124 (miR-124) is found able to suppress Rac1 mRNA and protein levels in pancreatic cancer cells, through the binding onto 3'-UTR of the Rac1 mRNA[16]. Although the suppression of RhoA by miR-124 is yet to be determined; miR-143 is found able to suppress Rac1 and RhoA at the same time, producing a decreased tumour migration result in a pancreatic tumour cell xenograft model[18].

From the recent findings, Rac1 is difficult to target and obtain therapeutic value, owning to switching on another oncoprotein RhoA, however, the discovery of miR-143 is exemplifying; microRNA could be the way out in tackling target that is similar to Rac1 which has an antagonist carries the similar tumour proliferative and metastasis function. It is worth to investigate on how miR-143 suppressing this "double fused" system in pancreatic tumour metastasis enhancement and its effect on long term survival.

RalGEF-Ral effector signaling network

Ras-like guanine nucleotide exchange factors (RalGEFs) and Ras-like (Ral) protein (which is also named as Ral small GTPase) have drawn increasing attention in cancers mediated by Ras, because RalGEFs are one of the direct effectors of activated Ras[19] and the discoveries of the important roles of Ral proteins in tumourigenesis and metastasis[20], however, the exact mechanism of the signaling network requires further studies to complete. There are more than four kinds of RalGEFs (*e.g.*, Ral-GDS, Rgl1, Rgl2, and Rgl3) and two homologues of Ral are found, Ral-A and Ral-B, in which they share same nucleotide sequence but differ in 82% of amino acid sequence[19-21]. It is known that activated Ras will activate RalGEFs and in turn the activated RalGEF will convert the GDP bound Ral into GTP bound Ral, the activated Ral GTPase will then activate its downstream targets, for instance RalBP1, filamin, PLCδ1, PLD1, *etc.*, bringing

out the corresponding biological responses[19]. Although the general mechanism is elucidated nowadays, the exact RalGEFs activating RalA and RalB are remain unknown, so do the identity of the exact Ras proteins in activating a particular RalGEFs[19].

There are two homologues of Ral small GTPase which are named RalA and RalB, their roles are distinct in tumourigenesis[19], but are seemingly overlapped in metastasis and invasiveness[21], ubiquitinated form of RalA and RalB have been found and it is in a non-degradative manner for selective localization modulation and functional regulations of Ral[22]. Studies have shown that mutated RalA in a constitutively active state can cause transformation of human cells but not in the same mutant of RalB[23], stable suppression of RalA in pancreatic cancer cells has brought a significant inhibition in the anchorage-independent growth[19], and inhibition of RalA can delay the tumourigenesis K-Ras mutants PDAC in mouse model[19], and the binding of RalA onto RalBP1 or Sec5 is found crucial in Ras - mediated transformation[23]. Aurora A kinase (AAK) is a kind of RalA inhibitors which prevents RalA phosphorylation, in fact an AAK, MLN8237 has been entered phase Ⅲ clinical trials, and such targeting is not effective in suppressing RalA signaling[19].

On the other hand, suppression of RalB alone does not reduce tumourigenesis and transformation but bringing a more pronounced effect in metastatic tumour growth suppression when compared to RalA inhibited alone pancreatic cell lines. In addition, enhanced apoptosis in RalB suppressed cells in suspension state[24]; these are suggesting RalB has a more significant role in the control of metastatic growth of cancer cells than RalA[21]. However, when abrogating the expression of either RalA or RalB in pancreatic cell lines, reduced invasiveness is observed in some pancreatic cancer cells with RalA or RalB suppression but not in all kinds of pancreatic cancer cells, *e.g.*, reduced invasiveness is observed in RalA and RalB suppressed Capan-1 cell line, while in Panc-1 cell line suppressed RalA boosted the cancer cell invasiveness and RalB can bring a reduced invasiveness, and in T3M4 cell line RalB suppression cannot bring down the cancer cell invasiveness but RalA suppression can bring a reduced invasiveness[21]. Thus, RalA and RalB may participate in the control of the invasiveness of pancreatic cancer cells, but there should be some other signaling pathways in control to this tumour malignancy, as the invasiveness reduction cannot be observed in all types of pancreatic cancer cell lines[21]. In regard to the observations, RalB has been suggested in maintaining the viability of the cancer cells in the circulatory system and ensuring tumour cells invasiveness to other organs[21].

Nevertheless, the localization of Ral proteins may also have their roles in the control of the cancer malignancies and is in relation to their ubiquitination and phosphorylation status, as de - ubiquitination of RalA in lipid raft microdomains is reported at the loss of cell-matrix interactions, and ubiquitination of RalA promotes lipid raft microdomains exposure on the cell membrane

when the cell got re-adhered[22]. Since lipid raft microdomains served as the platform for various signaling pathways, when cancer cell is in detached state, its growth is inhibited due to the loss of related signaling cascade in the lipid raft microdomains[24,25]. Therefore, the prevention of the re - exposure of the lipid raft microdomains onto the membrane can be a direction in the RalGEF - Ral signaling cascade for inhibiting the cancer metastasis by targeting the ubiquitination and activation of RalA.

All in all, the Ral proteins in the RalGEF - Ral signaling cascade play important roles in the control of cancer malignancies, targeting the RalGEF would seem to be efficient in shutting down the transduction of the signaling cascade, however, the question of the availability of inhibitors to RalGEF is concerning, as it is a Ras like signaling molecules, the design of an effective inhibitor to RalGEF may not be easy, and the effectors downstream of this signaling cascade should be closely investigated to aid the discovery of inhibitors that can block the signal transduction downstream of this pathway.

SIGNALING PATHWAYS

Notch signaling pathway

Notch signaling is found to be an important pathway in pancreas development, however the exact mechanism of how Notch regulates pancreatic development and the effectors it recruits are not fully understood[26]. Notch signaling pathway has been reported to maintain a pool of pancreatic progenitor cells at the early stage of pancreatic development, and governs pancreatic ductal cell differentiation which found to be triggered by the intensity of the Notch activation[26]. Implying Notch signaling mediates different effectors depends on cell type, and the stage of organogenesis.

In the pancreatic cancer, Notch signaling molecules are over-expressed[26] and could produce oncogenic, anti-tumour, and drug resistance[27] activities basing on the cellular context[26]. In an ADM study using mouse PDAC model has shown that subject carrying mutant KRAS[28], Notch is constitutively activated and up-regulated in the absence of EGFR[28], while in wild type KRAS carrier, Notch activation requires EGFR activation to induce ADM[29]. Implying the mutation of Ras could alter the activation pathway of Notch. Moreover, the anti-tumour activity of Notch signaling is brought out by Notch2 receptor deletion in mutant KRAS carrier[29], which showed PanIN development is inhibited and subject survival is risen[29]. For the same model, deletion of Notch1 resulted an opposite effect, PanIN development is accelerated and subject median survival is decreased[28,30]. As these two Notch receptors are localized in different compartment of a pancreatic cell, and the exact location of them is not yet concluded[26]. Thus, studying the distribution of Notch1 and Notch2 in pancreatic cell may help to understand their roles in pancreatic cancer and the effectors downstream of this signaling pathway.

As the functions of the Notch1 and Notch2 receptors appeared to be distinct and the involvement of EGFR for activation, the roles of Notch receptors may act as the decision maker in deciding how the cell behave according to the external environment. Due to the complex environment during cancer development, figuring out the roles of Notch at each stage of the pancreatic cancer development will definitely help sorting out targets this signaling pathway that can compromise pancreatic cancer.

Nodal/Activin signaling pathway

Nodal and Activin are morphogens which are being secreted into extracellular region[31,32] to mediate gene expression in target cell through phosphorylating the transcription factor mothers against decapentaplegic homolog 2, 3 and 4 (Smad2, Smad3, and Smad4)[33], and the signal intensity is found to be able to determine the cell fate decision that the target cell would execute[33]. Thus it is an important switch in deciding cell differentiation, self-renewal and pluripotency maintenance[33], the decision of the cell fate control is found to be related to the signal intensity and signal gradient generated by this pathway[33].

It is found that these two morphogens are over-expressed in pancreatic stem cells and pancreatic stellate cells, their expression levels are barely detectable in highly differentiated pancreatic cancer cell and normal pancreas or other developed tissues[34]. Moreover, it has been suggested that a small population of cancer stem cell is encompassed in pancreatic carcinomas[34], therefore, taking these two characteristics together this signaling pathway can be a specific therapeutic target for pancreatic cancer.

The common receptors of Nodal and Activin which are named Activin-like type I receptor 4 and 7 (Alk4 and Alk7, also written as Alk4/7), are being targeted by the inhibitor SB431542[34]. Targeting Alk4/7 is to abrogate the signal transduction from Nodal/Activin receptors to the transcription factors Smad 2, Smad 3, and Smad 4; and preventing the downstream genes transcription which favor tumour malignancies expression[34]. Under *in vivo* condition, pancreatic cancer cell L3.6pl pre-treated with co-administration of SB431542 and gemcitabine before implanting onto immunocompromised mice, have resulted a significant increase in apoptosis of cell carrying CD133$^+$ surface marker, implying such regimen can deplete the population of cancer stem cell in pancreatic cancer, and prevented the tumorigenicity of the cancer cell in this xenograft model; while such observations cannot be obtained in either single treatment alone[34].

However, such regimen is challenged by the abundant stroma in the xenograft model employing primary pancreatic cancer tissue, co-administration of SB431542 and gemcitabine cannot inhibit the tumour growth in such model, overcoming the sheltering effect of stroma to the pancreatic cancer cell is vital for efficient drug delivery to the tumour cell[35]. The triple-administration of SB431542, gemcitabine and CUR199691 resulted in an enhanced depletion of cancer stem cell population, as CUR199691 is an inhibitor targets hedgehog signaling pathway and ultimately depletes the stroma[34].

Besides, pancreatic tumour cells with certain mutations on Smad 4 gene have showed to be less responsive towards the regimen[34]. As Smad 4 is one of the factors for the signal transduction in the Notch/Activin signaling cascade[34], thus it is essential for the future studies to identify others up-stream targets controlling mutated Smad 4, so as to provide regimen for pancreatic cancer patients with mutations in Smad 4.

Nodal/Activin signaling is a promising target in elimination of pancreatic cancer stem cell from the studies presented here, despite its limitation in pancreatic cancer patients with mutations in Smad 4 gene, its effectiveness in wild type Smad 4 still makes it an attractive target in primary pancreatic cancer tissue model with the use of hedgehog inhibitor.

Metastatic suppressor-N-myc downstream-regulated gene-1

The N-myc downstream-regulated gene-1 (NDRG1) has recently been identified as a metastasis suppressor in several human cancer types[36], including human pancreatic cancer[37]. NDRG1 is found to increase the expression of tumour suppressor gene Smad4, which further inhibits the phosphatidylinositol-3 kinase (PI3K)/phosphorylated protein kinase B (AKT) signaling and extracellular signal-regulated kinase (ERK) pathway[36], besides, NDRG1 inhibits broad signaling molecules in nuclear factor - kappa B (NF-κB) signaling pathway, which resulted in reduced cancer metastasis[37]. As these three signaling pathways contribute to cancer cell proliferation and metastasis promotion, and cross-talk activities among them[36], therefore, NDRG1 is playing a role of modulator in orchestrating the signals in this triad networks.

The regulation of NDRG1 is debatable; numerous of studies have found out that hypoxia condition[38], epigenetic regulation[39] and iron depletion[40] can up-regulate NDRG1 expression level and such up-regulation seems to correlate with the differentiation status of the cancer cell.

It is worth to note that in a human PDAC model, under 2% of oxygen supply the NDRG1 mRNA and protein levels are elevated in differentiated pancreatic cancer cells but there are no change in the mRNA and protein levels in poorly differentiated cell lines[38]. Suggesting NDRG1 expression depends on both hypoxic condition and differentiation status of the tumour cell. The main focus would be on the rationale behind this phenomenon, as undifferentiated pancreatic cancer cell is comparatively more invasive and metastatic than highly differentiated counterpart[38]. In light of this, poorly differentiated pancreatic cancer cell (*e.g.*, Panc1) would have its NDRG1 level being suppressed in order to maintain high CXC chemokines[37] and high pro-angiogenic factor vascular endothelial growth factor (VEGF) expression[38] to direct cancer cell proliferation and angiogenesis. While NDRG1 over - expression has found down-regulating of these two signaling molecules and leading to suppression of tumour growth and angiogenesis[37].

The low NDRG1 expression in undifferentiated pancreatic cancer cells is related to the epigenetic regulation, as treating the undifferentiated pancreatic cancer cells with methyltransferase inhibitor 5-aza-2'-deoxycytidine has enhanced NDRG1 protein expression level, however, the epigenetic control on NDRG1 is not directly acting on the NDGR1 promoter, as there is no significant DNA methylation in the NDRG1 promoter region; suggesting other genes being silenced are essential for the NDRG1 expression[39].

As NDRG1 expression is affected by numerous factors, studies of targeting the molecular events downstream of NDRG1 are carried out, for instance an novel synthetic derivative of curcumin (CDF) has shown its inhibitory effect of the expression of VEGF, hypoxia inducible factor-1 α (HIF-1α), miR-210 and cancer stem cell self-renewal properties under hypoxia condition and are crucial for pancreatic cancer cell to promote tumour angiogenesis[41].

Although the exact mechanism of controlling the NDRG1 remains unclear, the current findings have suggested maintaining a high NDRG1 expression level in undifferentiated cell is able to suppress the tumour malignancies. Therefore, studies in finding enhancing NDRG1 expression genes is important in suppressing pancreatic cancer growth and metastasis.

Energy metabolism

As mentioned in the previous sections, the low vascularity structure of pancreatic tumour leading to a hypoxic environment and the adaptation of pancreatic cancer cells in hypoxic conditions through enhanced proliferation, angiogenesis and metastasis have been described. However, the primary element for cell survival is energy source which normally generated in glycolysis and Kreb's Cycle, as pancreatic cancer cells have an oxygen scarcity issue[42]; metabolic changes in pancreatic cancer cells allow them to cope with hypoxia.

First, the utilization of glucose would rely heavily on TCA-independent pathways, for example, there is up-regulation of pentose-phosphate pathway, anaerobic respiration for ATP production in hypoxic environment[43]. Secondly, glutamine metabolism is also elevated in hexosamine biosynthetic pathway which is crucial for the production of UDP-N-acetylglucosamine, and it is used for glycosylating proteins in proteins modification[44]. Glutaminolysis is also employed by hypoxia pancreatic cancer cell which metabolizing glutamine to generate glutamate and can be further metabolized in TCA cycle to produce pyruvate and lactate for further ATP production[44]. Lactate production is important for tumour cell invasiveness and neighboring cell proliferation, as inhibition of the enzyme glutamine fructose-6-phosphate amidotransferase by azaserine can cause significant reduction in hypoxic pancreatic cell proliferation[42]. Apart from targeting glutaminolysis, cannabinoids are found to suppress TCA cycle and induce the reactive oxygen species which leads to AMP-activated protein kinase

level increase to mediate autophagy in pancreatic cancer cells[45-47]. The ROS signaling activation could also abrogate the electron transport chain in mitochondria with unclear mechanism[48], leading to depletion of ATP in the cell and the AMPK dependent autophagy would be mediated[45].

By considering the founding in the energy metabolism of PDAC, targeting glutamine, glucose metabolism and increase the ROS production in hypoxic region in pancreatic tumour can elicit autophagy in PDAC. It is important to evaluate the effects of targeting them in *in vivo* model, as blocking major metabolic pathways is very likely to damage normal tissues, specific targeting the metabolic pathways in PDAC would minimize such drawback and enhancing the therapeutic value of targeting the energy metabolism pathway.

RECEPTOR PROTEINS

DR5
The death receptor 5 (DR5), is found to be frequently expressed in pancreatic cancer stem cell[49] and mediates cancer cells apoptosis *via* caspase 8 recruitment upon interacting with another receptor, Tumour necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL)[50,51], forming the death-inducing signal complex (DISC) to induce apoptosis, thus this enables the elimination of pancreatic cancer stem cell specifically, and reduces the occurrence of tumour relapse and overcoming the chemoresistance of pancreatic cancer stem cell[52].

Several studies on targeting activation of Apo2L/TRAIL induced apoptosis have been carried out, by combing with chemotherapies to obtain a synergistic effect in shrinking cancer stem cell population in pancreatic cancer[53]. Co-administration of DR5 agonist Tigatuzumab and gemcitabine has recorded more tumour regression on PDA xenografts than administrating either agent alone[49]. Moreover the up-regulation of several signaling molecules, for instance, cell surface death receptor Fas, Fas-associated death domain, and tumour necrosis factor receptor 1-associated death domain in the apoptotic pathway are also recorded[49]. Indicating the co-administration of Tigatuzumab and gemcitabine can result in cell growth inhibition and apoptosis for cell expressing DR5[49].

Another study has showed that dihydroartemisinin can increase intracellular ROS concentration and would lead to DR5 expression elevation and in turn mediate apoptosis *via* Apo2L/topoisomerase, TNF-α-related apoptosis-inducing ligand (TRAIL)[52]. Revealing the apoptotic pathway activation through DR5 requires high intracellular ROS[52]. Therefore, eliciting apoptosis in DR5 over-expressed cancer cell is a promising therapy for pancreatic cancer[50].

PAR2
The Protease-activated Receptor-2 (PAR2) is a member of the G-protein coupled receptor family and is acti-

vated by trypsin[54]. PAR2 is able to promote angiogenesis through two distinct pathways. The first one is *via* the activation of the mitogen-activated protein kinase (MAPK) to mediate VEGF release[55], another pathway involves the tissue factor to bind with integrin-linked kinase to up-regulate HIF-1α expression *via* AKT phophorylation and eventually enhanced VEGF expression[56]. Hence, PAR2 is essential for tumour survival under hypoxic condition in the micro environment, as PAR2 maintains a constitutive high level of HIF-1α for angiogenesis promotion and this also explains the high metastatic property of pancreatic cancer cell in hypoxia region.

Besides, the role of PAR2 in pancreatic cancer cell migration is also being reported, PAR2 is found to mediate MAPK-epidermal growth factor receptor 1/2 (EGF1/2) signaling pathway with the utilization of extracellular ATP, blocking the cross talk between PAR2 and extracellular ATP can be a target in reducing pancreatic cancer metastasis[57].

HER3
The Human Epidermal Growth Factor Receptor (HER) family consists of four members in which they are all type 1 transmembrane receptor with tyrosine kinase properties[58], except HER3[59], a member of HER which is found overexpressed 41% in pancreatic cancer[60]. Because of lacking tyrosine kinase activity in HER3, it requires phosphorylation by another HER receptor to activate PI3K/AKT signaling pathway to mediate cell angiogenesis and metastasis[61]. The expression level of HER3 has been correlated with tumour progression[59].

Therefore, HER3 has been an important target for suppressing tumour angiogenesis and metastasis by using humanized monoclonal antibodies, *e.g.* U3-1287 which has gone through phase 1 clinical trials with well tolerance in solid tumour patients , MM-121 and tyrosine kinase inhibitors[62,63]. The anti - HER3 agents block the activation site on the HER3 receptor, preventing the activation of HER3 during heterodimerization with HER2 receptor[59] and promoting receptor internalization upon binding onto its extracellular domain[58]. This reduces the activation of PI3K/AKT signaling pathway and its downstream effectors activation, resulting tumour growth suppression[59].

In view of this, because of HER3 over-expression in PDAC and its crucial role in activating the signaling pathway essential for cell growth, it is a valuable and specific therapeutic target upon co-administration of anti - HER3 agent and gemcitabine has resulted an enhanced anti-tumour effect[64], confirming the therapeutic value of anti - HER3 agents in PDAC and is worth investing in more clinical studies.

All in all, we have described three receptor proteins which carry out apoptosis, tumour proliferation and metastasis. Current studies are focusing on how to trigger the signaling molecule that could induce apoptosis, inhibit the receptors that favor cancer proliferation and metastasis, so as to reduce tumour progression. How-

ever, the possibility of combining these two approaches in the same model is not yet published, in which the total effect on tumour clearance is expecting to be more efficient.

CELL SURFACE PROTEIN

E-Cadherin

E-Cadherin is a transmembrane protein[65] and is a member of cadherins family in which its expression in epithelial cells is controlled by intracellular signaling molecules[66]. E-Cadherin directs the positioning of the cell during morphogenesis, controlling cell migration and tissue structure maintenance[67]. It is reported that during epithelial-mesenchymal transition (EMT) the E-Cadherin level in neoplastic epithelial cells is down-regulated, suggesting triggering the dedifferentiation of neoplastic epithelial cells into mesenchymal cell with higher motility[65].

The fading E-cadherin expression is frequently reported in undifferentiated, noncohesive pancreatic cancers[68], and it is found that the silencing of E-cadherin is mediated by Snail/ histone deacetylase 1 (HDAC1)/histone deacetylase 2 (HDAC2) complex[65] and Enhancer of Zeste Homolog 2 (EZH2)[69] through hypermethylation of its promoter region[68]. Inhibition of Snail and HDAC2 are also carried out to validate the E-cadherin expression is under such complex governance[70].

Since the absence of E-Cadherin marks the onset of metastasis and PDAC progression, a study targeting E - Cadherin restoration by using microRNA 101, has inhibited the EZH2 binding on E-Cadherin promoter region in PANC1 preventing E - Cadherin silencing and in turn inhibited its tumorigenicity xenograft[69].

The key for targeting E-Cadherin to obtain therapeutic value in PDAC is to up - hold the E - Cadherin expression by down - regulating its inhibitor, as described above, inhibiting EZH2, and Snail/HDAC1/HDAC2 can reduce E-Cadherin depression and suppresses the tumorigenicity of pancreatic cancer, in the future studies, discovering targets that suppress E-Cadherin expression is important for therapy involving E - Cadherin.

Galectin-4

Galectin-4 (Gal-4) is a glycan binding proteins which belongs to the galectin family. Gal-4 is found over-expressed in cystic tumours of the human pancreas, PDAC and cancer stromal cell[71]. Activated galectins carry out several functions; include cell-cell adhesion, cell proliferation[72], mediation of intracellular signaling[73] and tumour metastasis[74], *etc.*, However the mechanisms behind are remain unknown.

A study has evaluated the inhibition effect of Gal-4 in a pancreatic cancer cell, PaTu-S cell, can lead to enhanced tumour migration[74]. The exact reason is yet to be elucidated, but it is speculated that the reduction of Gal-4 on the cell membrane would destabilize cell-cell interaction, allowing tumour cells escape[75]. Another important implication suggests Gal-4 expression may be dependent on the tumour development stage, and is vital for tumorigenesis as it promotes cell-cell adhesion[76].

Because of Gal-4 multi-roles in expressing tumour malignancies, and the little knowledge on how Gal-4 control cell migration and tumour metastasis, it is worth to investigate its related signaling pathways and identifying possible inhibitors so as to enable targeting Gal-4 in treating pancreatic cancer.

TMPRS S4

Transmembrane Protease, Serine 4 (TMPRSS4) is found highly expressed in several cancer cells, including pancreatic cancer cell[77]. However its regulation mechanism is poorly known[78], several studies have shown that TMPRSS4 can promote EMT, metastasis and invasiveness in human epithelial pancreatic[79], lung and colon cancer[77].

It is reported that EMT mediation is not solely rely on TMPRSS4 up-regulated integrin $\alpha 5$ to activate FAK/ERK signaling pathway and enhanced invasiveness[79] but also count on the down-regulation of E-Cadherin in TMPRSS4 up-regulated cancer cells[79].

Another downstream target of TMPRSS4 is the urokinase-type plasminogen activator (uPA) gene[80]. TMPRSS4 would activate the transcription factors of μPA *via* c-Jun N-terminal kinase mechanism before promoting μPA transcription in a cell-type dependent manner[80]. And the increased μPA gene transcription marks the increased tumour cell aggressiveness.

The coupling effects of TMPRSS4 on tumour aggravated invasiveness and metastasis with other signaling molecules (*e.g.*, integrin $\alpha 5$, uPA), moreover, the inverse expression pattern of TMPRSS4 and E-cadherin suggests TMPRSS4 can be suppressed by targeting E-cadherin inhibitors as previously mentioned and should be investigated in future studies. TMPRSS4 expression is affected by various signaling molecules and by considering its important role in expressing tumours malignancies, it is a target with multiple approaches for suppression.

IAP

Inhibitor of apoptosis protein (IAP) is a group of proteins bind to caspases and inhibit caspases apoptotic effect resulting apoptosis abortion[81]. The importance of apoptosis mediation in cancer therapies has an irreplaceable place, and therapies incapable to induce cell death would be meaningless. However, most therapies nowadays involve the elicitation of apoptosis at their end, and resistance of the corresponding therapies developed due to the presence of IAP[82]. Therefore IAP is the obstacle to tackle with, so as to ameliorate the effectiveness of therapies targeting apoptosis induction.

Two of the IAP members would be discussed here which are X-linked IAP (XIAP) and survivin, because of the reports of their close interaction in triggering anti-apoptotic effect[82].

Survivin's action has been controversial in anti-apoptotic activity[82], it is reported that survivin carries out neurogenesis, angiogenesis, cell cycle progression in can-

cer cell[83] and displays caspase inhibitory effect through associating with XIAP and stabilizes XIAP *via* their baculovirus-inhibitor of apoptosis repeat (BIR) domain[84]. Most of the survivin inhibitors that are under clinical trials have improved the effectiveness of chemotherapies *e.g.*, topoisomerase, TRAIL[82]. Revealing survivin's submissive and supporting role in IAP targeted treatment.

XIAP is the most studied IAP, it is found able to suppress caspase-3, caspase-7 and caspase-9 apoptotic activities[85]. Inhibition of its caspase binding domains, which are named, BIR-2 and BIR-3, with the use of phenylurea-based chemical inhibitors of XIAP (XAntags) could make pancreatic cancer cells more vulnerable to apoptosis[85].

Because of the anti-tumour effect in inhibiting XIAP and the supportive role of surviving in apoptotic inhibition, co - suppressing XIAP and survivin has also been performed in Panc-1 cell[86], resulting cell proliferation hindrance, and enhanced gemcitabine effectiveness in XIAP and survivin suppressed model than sole suppression of either IAP[86].

Nevertheless, there is no cell toxicity recorded in XIAP knocked out mouse model and *in vitro* cell model, possibly by the compensatory up-regulation of other cIAPs, and the masking effect of such up - regulation requires further studies[81]. From the recent findings in IAP, inhibiting IAP is a promising therapeutic direction in promoting apoptosis progression in PDAC cells, thus enhancing the effectiveness of current chemotherapies upon co - administration in treating PDAC.

NON-CODING RNA

MicroRNA
MicroRNAs (miRNAs) consist of 18-24 base pair which are small and non-coding-sequence[87]. They execute target gene expression control by binding miRNA 3'UTR on to the target gene mRNA[87], and only when perfect binding of miRNA on to the target mRNA could mediate mRNA cleavage, otherwise, it would result into inhibited protein production[88]. miRNAs which induce over-expression of oncogenes are termed the oncogenic miRs (onco-miRs), on the other hand, miRNAs which suppress cell transformation are named tumour suppressor miRs (TSG-miRs)[89]. The abnormal expression levels of these two kinds of miRNAs are observed in pancreatic cancer[90]. Current studies are either suppressing onco - miRs or reconstituting the TSG-miRs level[91], therefore, in the following we will discuss some TSG-miRs which are promising therapeutic targets in pancreatic cancer.

miR-34: miR-34 is reported to be up-regulated by p53[92], inducing cell cycle arrest in primary and tumour derived cell lines[93]. A significant reduction of miR - 34 expression level in gastric cancer cells with p53 mutation has been observed and reconstituted miR - 34 expression by transfecting pancreatic cancer cells with letivirus carrying vector expressing miR - 34[94], and resulted in decreased

Notch2 and Bcl-2 protein production, reduced tumour-sphere formation from cancer stem cell (CSC)[94]. Although the relationship between miR-34 and p53 is still unclear[94], the encouraging results generated by miR-4 in p53 deficient pancreatic cancer cells[93] have make it a worthy therapeutic target.

miR-143: miR-143 has been studied for its anti-metastasis and anti-tumour proliferation in liver undergone metastasis and a pancreatic cancer xenograft in mouse model, respectively[95]. miR-143 expression level in KRAS mutant pancreatic cancer cells is also being ablated[96], re-expressing miR-143 in its deficit cell lines has performed, GEF, RAC1, matrix metalloproteases (MMPs) and KRAS are the inhibition targets for miR-143[95], as described previously lessened RAC1 level can inhibit metastasis and tumorigenesis, while inhibiting KRAS is even more important, which implies a board spectrum of signaling pathways diminishing effect.

Another tumour growth promotion factor that miR-143 targets is the cyclooxygenase (COX-2)[97], COX-2 is reported as an essential factor for prostaglandin synthesis to mediate inflammation and cancer cell growth and survival[98]. In pancreatic cancer cell, miR - 143 was found to be repressed by prostaglandin[99], and restoration of miR-143 level can decrease both mRNA and protein level of COX-2 and inhibited cell growth[98].

miR-200: miR-200 is a family of miRNAs related to EMT[100], reconstituted expression level of miR-200 has restored the phosphatase and tensin homolog (PTEN) expression level[100], as PTEN is widely down regulated in various cancer cell lines and is a tumour suppressor gene in which reduced expression would lead to enhanced tumour aggressiveness[101,102], while membrane type-1-matrix metalloproteinase (MT1-MMP) is up-regulated and lead to aggravated cancer invasion[101-103]. Restoration of miR-200 by using CDF, which is a synthetic analog of curcumin, and a natural compound, BR - DIM are reported and are found able to enhance PTEN expression level and a decreased MT1-MPP promoted invasiveness[100]. Therefore, agents which could enhance miR-200 expression would have promising therapeutic value in curbing pancreatic cancer aggressiveness for enhanced treatments efficiency.

The three TSG-miRs exemplified the diverse roles of miRNAs in anti-tumour activities, up-regulation of TSG-miRs can suppress tumour malignancies expression, however, suppressing onco-miRs that can up-regulate oncogenes also have tumour malignancies suppression effect, therefore screening and studying the agents that can up-regulate TSG-miRs and down-regulate onco-miRs are vital for PDAC therapy development.

Long non-coding RNA
Long non-coding RNAs (lncRNAs) are transcribed from intergenic and intronic regions in human genome[104] by RNA polymerase Ⅱ[105], which lengths more than 200 bp[106] and their biological functions have been reported,

for instance, epigenetic control, transcription regulation, pre and post-translational regulation[107], cell cycle and differentiation control and even governing the apoptosis process[108]. lncRNA is used as a diagnostic parameter and can be a therapeutic target in cancers[104]. However, the definition and discovery of lncRNAs are expected to keep on changing as very little is known in this emerging area[109]. In the following, two lncRNAs that are highly expressed in pancreatic cancer will be discussed.

HOTAIR: HOX transcript antisense RNA (HOTAIR) is a lncRNA which is highly expressed in a range of primary tumours and metastatic cell[110], in which its expression pattern is variable but in general is also over-expressed pancreatic cancer[111]. HOTAIR carries out tumour supporting effect by inhibiting anti - tumour genes activity, in which the interaction of HOTAIR and a Polycomb-group (PcG) family protein named, EZH2, would promote chromosomal histone protein histone 3 protein at lysine 27 (H3K27) trimethylation, which leads to repressed transcription of multiple gene targets[112]. However, there are some genes inhibited in an EZH2-independent manner[113].

Suppressed HOTAIR expression by using RNAi in pancreatic cancer cell has caused retarded cell growth, diminished tumour aggressiveness; altered cell-cycle progression and apoptosis induction[114]. Thus, relieving the repressed transcription of the tumour suppressor genes by suppressing HOTAIR expression is therapeutically valuable in treating PDAC. As the studies of genes activation mechanism and the genes that are targeted by HOTAIR are still ongoing[114], and the mechanism of genes being independently regulated by EZH2 but dependent on HOTAIR only, are currently under studies.

Studies of targeting HOTAIR in PDAC cell lines have achieved reduced tumour malignancies expression, indicating the relieved tumour suppressor genes expression by targeting HOTAIR has made HOTAIR an attractive target in pancreatic cancer therapies development. However, cautions should be taken on the over-expressed genes induced by HOTAIR, as HOTAIR induced and suppressed multiple genes at the same time and some of the over-expressed genes expression level do not reduce with HOTAIR suppression[114], suggesting another mechanism may exist in down-regulating them. In conclusion, a more thorough understanding on the regulation and the functions of HOTAIR induced and suppressed genes, it could lead to a more rounded target in promoting tumour suppressors genes functions while inhibiting oncogenes activities.

MALAT1: Metastasis-Associated Lung Adenocarcinoma Transcript 1 (MALAT1), as known as the Nuclear-Enriched Abundant Transcript 2[115] is found highly expressed in normal pancreatic and lung tissues with high abundance and highly conserved among mammalian[116]. Intensive studies of MALAT1 in non-small-cell lung carcinoma revealed its metastasis and tumorigenicity promotion activities[117]. Although inadequate studies of MALAT1 in

pancreatic cancer cell model, it has been reported for its promotion of tumour malignancies expression in various cancer types[116]. For instance, in colorectal cancer, a Chinese herb extract Resveratrol is shown to down-regulate MALAT1 and cause suppression of Wnt/β signaling *via* decreasing β-catenin nuclear localization and eventually inhibited the invasiveness and metastasis of colorectal cancer[118].

Apart from promoting tumourigenesis, invasiveness and metastasis, MALAT1 also participates in the control of cell cycle progression, oncogenic transcription factor B-MYB is found up-regulated with the expression of MALAT1, silencing of B-MYB in fibroblast model resulted into cell cycle arrest in G1/S and S phase[119], moreover, another transcription factor, E2F transcription factor 1 (E2F1), which is essential for cell cycle progression and is also under the modulation of MALAT1, however down - regulated MALAT1 brought down E2F1 expression has elicited p53 expression enhancement and lead to cell cycle arrest and hence reduced cell proliferation[119]. This implies MALAT1 could induce DNA damage response *via* an unknown mechanism[119].

With regard to the findings of MALAT1 in other cancer, MALAT1 expression in PDAC is also very likely to correlate to pancreatic cancer progression. Although MALAT1 expression level is high in normal pancreatic tissue, its expression level in pancreatic cancer is not yet reported and also the role of MALAT1 in pancreatic tumour activities. Thus, if MALAT1 has a similar tumour malignancies promotion role in pancreatic cancer as in other cancer types, it would be a promising therapeutic target for PDAC treatment development.

EPIGENETIC MODIFIER

HDAC

Histone Deacetylases (HDACs) are a group of four classes of deacetylases[120], each class of the enzyme contributes to different tumour malignancies expression, for example, as mentioned previously, HDAC1 is responsible for the acceleration of EMT and metastasis in PDAC[121], while HDAC2 would desensitize the PDAC towards DNA damage response and decreased prop - apoptotic proteins[122], however, only the third class, which is named the human hst proteins (SIRTs) did not respond to HDAC inhibitors (HDACIs) under current clinical trials[120], but a HDACI named Sirtinol is able to induce apoptosis with the administration of Sirtinol[121], and its effect is further enhanced with the co - administration with gemcitabine[123].

The exact mechanism of HDACIs in mediating antitumour activities remain further elucidation. However, studies have shown that it is not necessary for HDACIs to inhibit the expression of HDACs in mediating tumour suppression, for instance, a class I and II HDACI did not cause changes in the expression level of HDAC1, and other tumour suppressor genes but has shown reduced cell proliferation in cervical tumour cell[124]. Moreover, a class I and II HDACI inhibitor is found able to cause

a changes in the expression profile of class Ⅲ HDAC, SIRTs[121]. These evidences suggest the working mechanism of HDACIs involve complex molecular control. On the other hand, Rel/p65 (NF-κB) is found relating to the expression level of HDAC, for instance, over-expression of class I HDAC in pancreatic cancer cells, their expression level of NF-κB is also high, besides, a class I HDACI valproic acid (VPA) is studied and found to cause a decreased expression in pancreatic cancer cells which leads to enhanced PDAC apoptosis[125], in which over-expression of NF-κB has been reported for enhanced tumour growth, angiogenesis, chemo-resistance and metastasis[125]. Blocking NF-κB activity by VPA can obtain anti-tumour effect in such case[126].

The action of HDAC on gene silencing is mediated by deacetylating the histone proteins in the chromatin leading to chromatin condensation, resulting silenced genes transcription[121]. As the genes being silenced in cancer are mostly related to tumour suppressors, anti - apoptosis, and often resulted in drug resistance, therefore, targeting HDAC by using HDACI is believed to reduce the these tumour malignancies expression by suppressing the related signaling pathways of the PDAC and synergistically enhance the anti - tumour effect of current chemotherapy.

BMI1: B-Cell-specific Moloney murine leukemia virus Insertion site 1 (BMI1), belongs to the polycomb group (PcG) which represses transcriptional activity of various genes[127]. Over-expression of BMI1 in a board spectrum of cancer cells is observed, it strengthens tumour growth by providing anti-apoptotic activities and participate in tumour metastasis by up-regulating PI3K/AKT signaling pathway[127]. In *in vitro* experiment, PDAC with BMI1 suppressed using shRNA has shown enhanced cell death in response to gemcitabine treatment, a significant decrease for its cell surface markers CD44+CD24+ESA+, loss of self-renewal ability, reduced tumour sphere formation by CSCs and reduced tumour size in xenograft model[127].

Because of the diversified anti-tumour effects of silencing BMI1 in pancreatic cancer cell, such as reduced invasiveness, tumourigenesis[127], metastasis, CSC phenotypes, cell proliferation[128] and also chemo-resistance[127]. Besides, CSC is reported to be the causes for tumour relapse in pancreatic cancer[129], thus, the diversified roles of BMI1 in pancreatic cancer have made it a very attractive target, future studies targeting BMI1 inhibition and its downstream effectors would benefit PDAC treatment development.

EZH2

The polycomb repressor complex 2 member, Enhancer of Zeste Homolog 2 (EZH2) is a histone methyltransferase which is highly expressed in pancreatic cancer cells[130], EZH2 mediates tumour suppressor genes transcription inhibition through trimethylation of the H3K27[131], such as suppressing Rap1GAP expression in squamous car-

cinoma[132], E-Cadherin in pancreatic cancer[131]. Besides, several reports have suggested EZH2 suppresses miRNAs in contributing to pancreatic cancer progression, *e.g.*, microRNA-218 (miR-218), microRNA-26a[133,134], miR-218 is essential in suppressing tumour proliferation and metastasis in nude mouse model[133], EZH2 is believed to interact with two polycomb repressive complexes (PRCs), PRC1 and PRC2, and promoting the methylation of the target miRNA promoter region to silence the miRNAs expression in pancreatic cancer[133]. It is found that with the administration of EZH2 inhibitor, such as 3-deazaneplanocin A (DZNeP), can reduce EZH2 expression of EZH2 and rescued the expression of miR-218 leading to tumour malignancies reduction[133,135].

Apart from suppressing miRNAs in tumour progression, studies of the role of EZH2 as a tumourigenesis initiator have found that EZH2 also suppresses tumour suppressor gene p16^{INK4}, in which it suppresses tumour proliferation and regeneration, enhanced EZH2 expression has caused p16^{INK4} down - regulation, counteracting the suppression effects exerted by p16^{INK4}[136]. Such control is crucial for the regeneration of the injured acinar pancreatic cell, in which the injured cell undergone de-differentiation into metaplastic epithelial intermediate, depleted p16^{INK4} allows the cell to re-differentiate into acinar cell from metaplastic epithelial intermediate[136]. Thus in combination with the early appearance of PaIN lesion in pancreatic cell baring KRAS mutation and the loss of p16^{INK4} expression due to enhanced EZH2, invasive and metastatic tumour development are accelerated, demonstrating the linkage between regeneration and tumourigenesis under the influence of mutant KRAS[137].

Further studies of the role of EZH2 in pancreatic CSC has found it is essential in maintaining the CSC population in pancreatic cancer, suppressing EZH2 has decreased the degree of H3K27 methylation, reduced CSC population in pancreatic cancer, enhanced genes expressions for cell differentiation and migration[130]. Since the trimethylation of H3K27 and the expression is correlated with the CSC population, it is suggested that the H3K27 trimethylation by EZH2 can be used as a marker for the CSC population which allows rapid evaluation for the population of CSC when compared to conventional methods, hence, speeding up the studies of the effectiveness of compounds towards pancreatic CSC[130].

From the current findings of suppressing EZH2 in pancreatic cancer, EZH2 has an important role in tumour development initiation and supporting cancer stemness, and co-administration of DZNeP and gemcitabine has achieved promising anti-tumour effects. Nevertheless, EZH2 has also demonstrated its possibility to act as an indicator for CSC population estimation, and CSC elimination is an important factor for researchers to evaluate the efficacy of the compounds under studies, thus EZH2 is a versatile targets that possess both therapeutic and assay values and screening compounds suppressing EZH2 would definitely help speeding up therapies development

in PDAC.

PANCREATIC CANCER STEM CELL (PANCREATIC CSC)

The tumour cell population is reported to encompass a population of cancer stem cell (CSC)[138], and it is reported to give rise to the cancer stemness in various cancers, by carrying out self-renewal, metastasis, invasiveness enhancement[139], and drug resistance for pancreatic tumour[140]. Studies in CSC have led to the discovery of distinguishable cell surface markers presented in various cancer types, and this allowed the isolation of cancer stem cell for various studies[141]. In this section, we will briefly discuss how CSC contributes to enhanced cancer malignancies, and the plausible targets in CSC that have been reported to have therapeutic value.

Signaling pathways in CSC

There are three members of hedgehog proteins in the hedgehog signaling[141], a member of the hedgehog family, sonic hedgehog is found over - expressed in both pancreatic cancer cell and CSC[142]. The up-regulated sonic hedgehog signaling molecules facilitates the development of PanIN and enhanced accumulation of mutations in KRAS while inhibiting the hedgehog signaling pathway by the hedgehog signaling inhibitor cyclopamine has resulted decelerated tumour growth and on set of apoptosis[142], another inhibitor GDC-0449 is reported to produce reduced cell viability, caspase-3 mediated apoptosis, reduced tumour sphere formation in pancreatic CSC[143]. Sonic hedgehog has displayed its critical role in tumourigenesis initiation and tumour proliferation, targeting hedgehog signaling is therefore advantageous in the early development of pancreatic cancer.

In Notch signaling pathway, over-expressed Notch-1 promotes EMT and tumour sphere formation[144], which is confirmed by the increase expression of CD44 and EpCAM cell surface markers on CSC[144], suggesting Notch as a factor in pancreatic tumourigenesis in CSC, but the role of it in CSC self-renewal requires further studies[141]. Notch mediates signaling by nuclear translocation and is modified by γ-secretase before entering the nucleus, thus inhibitors of γ-secretase have been used to study the role of Notch in pancreatic cancer and also pancreatic CSC[145], a Notch inhibitor, PF-03084014 is found able to bring a reduction of CSC population, tumour re-growth and inhibited several cancer malignancies, *e.g.*, tumour growth, angiogenesis in pancreatic cancer xenograft model with the co-administration with gemcitabine[146], therefore its effect on pancreatic cancer is expectable.

The CXCR4 signaling pathway which comprises the ligand, stromal cell - derived factor-1/CXCR chemokine ligand 12 (SDF-1/CXCL12) and the G-protein coupled receptor, CXCR4. This signaling pathway is up - regulated in pancreatic cancer cell due to enhanced expression of CXCR4, and resulting into tumour metastasis promo-

tion; enhanced migration and strengthened stromal adhesion[141]. In pancreatic CSC, co-expression of CD133^{+} and CXCR4^{+} on the CSC signified a highly metastatic characteristic and contributes to tumour metastasis, therefore, disrupting the SDF-1 mediated CXCR4 signaling and depletion of the CD133^{+} CSC can abrogate the metastatic characteristic of pancreatic tumour[146]. Although targeting the CXCR4 in stopping pancreatic tumour metastasis looks promising, CXCR4 inhibitors are found highly toxic and non - specific reaction are the drawbacks that must have to be overcome before translating into clinical trials or practices[147].

Forkhead Box M1 (FoxM1) is a transcription factor found over-expressed in pancreatic cancer in which it promotes the expression of EMT characteristic[141], which is deduced by the increased mesenchymal cell markers expression including, zinc-finger E-box binding homeobox 1 (ZEB1), ZEB2, E-cadherin, and vimentin, and also enhanced tumour sphere formation which marks the strengthened self-renewal ability for CSC[148], as enhanced EMT is having close resemblance to CSC in giving rise to cancer stemness[149]. A natural compound genistein can inhibit the FoxM1 signaling pathway by down-regulating the expression of FoxM1 and its downstream gene targets (*e.g.*, VEGF, MMP-9) leading to reduced EMT and reduced tumour sphere formation and have resulted into reduced tumour growth and enhanced apoptosis[150]. The exact mechanism of the regulation of genistein on FoxM1 and its target genes is not clear yet, however, the application genistein can rescue the microRNA-200 (miR-200) expression by attenuated FoxM1 expression, and enhanced expression of miR-200 can inhibit the EMT progression[147]. Because of the important role for FoxM1 plays in the EMT and CSC progression, and the availability of FoxM1 inhibitor has made FoxM1 an attractive target and should evaluate the anti - tumour effects under co-administration of genistein and gemcitabine.

Cell surface marker on CSC

There are several cell surface markers on CSC which are not only be used to isolate CSC, but also have important functions towards CSC. For instance, expression of CD44^{+}/CD24^{+}/ESA^{+} mark the pancreatic cancer cell that function as CSC, with several signaling pathways (*e.g.*, BMI1, sonic hedgehog) up-regulated and self-renewal and tumorigenesis enhancement are observed[151], while ablated CD133 would lead to loss of CSC self - renewal ability[146]. This demonstrates the markers presented on the CSC can provide some clue on the de-regulated signaling pathways in the tumour which can help deciding the targets of the sub-population of the pancreatic tumour. Nevertheless, a novel CSC marker, c-Met, is found to be essential for tumour growth, tumour sphere formation and metastasis, inhibiting the expression of c-Met have suppressed these tumour malignancies[152], possibly *via* the downstream signaling pathways of c-Met, such as Ras-MAPK, and PI3K-AKT[153]. With the emerging knowl-

edge of the cell surface markers and their downstream signaling, options for targeting signaling transduction in PDAC is ever growing, besides, the surface markers can also act as the reference reflecting the de-regulated signaling pathways, hence, facilitating the therapeutic direction formulation.

TUMOUR MICROENVIRONMENT

Matrix metalloproteinase

MMPs are a group of zinc-dependent endopeptidases which hypothetically can degrade almost all proteins in the extracellular matrix (ECM)[154]. MPPs are over-expressed in pancreatic cancer and the biological roles of MMPs in cancer are to digest the proteins in basement membrane in the ECM which leads to enhanced migration of tumour cell[155], an evidence of migration signal mediation by cleavage of laminin-5 in ECM has been reported[156]. Moreover, cleavage of E-Cadherin by MMP-3, MMP-7[157] and A disintegrin and metalloprotease 10 (ADAM10) are observed, the loss of E-Cadherin not only enhanced cell mobility but also enhanced the tumour invasiveness and migration[158]. In addition, the release of pro-angiogenic inflammatory cytokine (TNF-α)[157] and VEGF[159] are correlated with the MPPs activity and all these confirm the role of MPPs in EMT and tumour metastasis promotion[160].

MMP is found related to the pancreatic stellate cell, which will be described in the next section, the TGF-α up-regulation in pancreatic stellate cell correlates with up-regulated MMP-1, suggesting a possibility of the association of these two molecules overexpression in enhanced tumour cell invasion[161].

As the importance of MMPs in tumour angiogenesis and metastasis is undeniable, studies of formulating MMP inhibitors (MMPIs) are undergoing; a MMPI, SB-3CT is able to reconvert the MMP-2 into its pro-enzyme state and has brought down liver metastasis[162]. However due to the usual late stage discovery of pancreatic cancer in real life[163] the use of MMPIs is limited and also MMPs activities have been observed to be stage dependent[164], therefore MMPs can be targeted for PDAC patients with early detection and can be applied widely when early detection method for PDAC is developed.

Pancreatic stellate cell

Pancreatic stellate cell (PaSC) resides in the exocrine of the pancreas and has dual roles in normal pancreatic tissue[165], first it acts as a storage of vitamin A[166], secondly upon pancreatic injury, the PaSC would be activated to acquire a myo-fibroblast-like phenotype which is called activated PaSC[167-169], activated PaSC will secret proteins into the ECM[170] resulting into pancreatic fibrosis[165] and on setting chronic pancreatitis which could lead to high risk of PDAC development. As mentioned in 10.1, high TGF-α expression level correlates with the high MMP-1 expression level in inducing PaSC migration, inhibition of MMP-1 has showed such migration induction is curbed by using MMP-1 tissue inhibitor and siRNA of

MMP-1[161], indicating PaSC activity can be modulated *via* MMPs.

Other studies by targeting PaSC proliferation and migration have been carried out, transgelin has been reported to be over-expressed in activated PaSC but not in normal acinar cell which could cause pancreatic fibrosis[171]. Moreover, knocking down transgelin expression has reduced cell proliferation and migration abilities in *in vitro* experiment[171], providing a biomarker for specific therapeutic target in knocking down PaSC population in the future.

Hedgehog signaling pathway

The Hedgehog (hh) signaling pathway, involves the secreted signaling molecules hedgehog proteins, which is classified into 3 subcategories, namely, Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh)[172]. Among these 3 hh, Shh is found over-expressed in 70% of primary PDAC[173]. Hedgehog biological roles have been described as an essential factor in embryonic development and regulate cell proliferation[172].

The mediation of hedgehog signaling is triggered upon the binding of Shh to the Patched 12-transmembrane domain receptor (Ptch) which further activates another transmembrane signal transducer, smoothened (Smo), that would lead to localization of transcription factors in the nucleus and initiate transcription of downstream effectors[174], for instance, Cyclin D2, FoxM1, jagged 2 (JAG2), *etc*[175].

It is reported that tumourigenesis and tumour proliferation requires constitutive activated hedgehog signaling, and in pancreatic stromal cell in PDAC, Smo is over-expressed and direct tumour cell growth in the vicinity of stromal cell, leading to a therapy targeting hedgehog signaling in tumour-stromal interaction[172]. Besides, report of Shh activation in CSC is crucial for CSC proliferation[176], and it has been discussed for the CSC in aggravating pancreatic cancer treatment, such as heightened drug resistance and tumour relapse. Therefore, studying hedgehog signaling inhibitors is beneficial for pancreatic cancer treatments. A hh signaling inhibitor, Sulforaphane has been found to inhibit self-renewal capacity in CSC *via* Shh signaling inhibition leading to downstream effectors *e.g.*, Nanog and Oct-4 suppression[176]. Moreover, inhibition of hedgehog in pancreatic cancer cells and tissue are performed and it is found that a marked decrease in EMT with EMT related transcription factors (Snail and Slug) down-regulation and had suppressed PI3K/AKT signaling, which is downstream of hedgehog signaling with an association of decreased cell proliferation[177].

Because of diversified roles of hedgehog signaling in tumour malignancies and CSC population maintenance, and the cross talk among other signaling pathways, *e.g.* FoxM1, Notch (*via* JAG2), targeting hedgehog may have a centralized effect in weakening the malignancies of pancreatic tumour.

Stromal environment-hyaluronan

The microenvironment of pancreatic cancer, has been

accused to be the major challenge in drug delivery because of its highly dense ECM, the penetration of even small drug molecules gemcitabine is prevented[178]. Besides, stromal cells which are the activated fibroblasts and PaSC, inflammatory cells[178]; and distorted vascular structure of blood and lymphatic vessel composed the stromal environment[179]. And the production of stroma is mediated by various factors involved in numerous signaling pathways in an autocrine and paracrine action, TGF-β, insulin growth factor 1, and EGF are the examples[180].

Among the molecules in ECM, hyaluronan or hyaluronic acid (HA) is secreted by PDAC[181], and is a repeat of N-acetylglucosamin/glucuronic acid disaccharide[170]. It is able to interact with a hyaladherin, CD44, to regulate tyrosine kinase receptor and to facilitate angiogenesis, EMT, and chemoresistance[182]. It is also one of the main components that contributes to high intra-tumoral fluidic pressure (IFP) through solvating with water molecule, hence, impeded the diffusion of drug molecules into the target tumour cell[183]. It is found that co-administration of hyaluronidase with gemcitabine or other drugs prolonged the localization of the accompanied drug in the tumour[183]. Therefore, it would become a trend for future development of drug target, *e.g.*, well incorporated with hyaluronidase to facilitate drug delivery, or even HA can be a target to disintegrate the condensed ECM, enhancing the responsiveness of the tumour cell to the treatment.

CONCLUSION

A range of therapeutic targets in PDAC have been briefly described in this article, in which their anti - tumour and oncogenic activities are characterized through various experiments and can be taken as potential target for PDAC therapies development.

Nevertheless, numerous of the targets are found overlapped with each other in producing certain kinds of tumour malignancies, *e.g.*, over-expression of CXCR4, Rac1, BMI1, and *etc.*, in pancreatic tumour cell have observed a metastasis enhancement. In light of this, and hypothetically, in order to prevent metastasis, suppressing these targets should have a more pronounced effect in metastasis inhibition. Moreover, such outflanked approach may also prevent the tumour cell from switching into other signaling pathways producing the same tumour malignancies, and achieving elimination ultimately. Besides, the current knowledge of each of these targets is insufficient, categorizing these targets by the tumour malignancies produced and identify if there is any relationship between them and understand the mechanism behind, would allow the discovery of linkages among them in terms of proteins and mRNA expression levels and mechanistic studies.

Last but not least, screening of suitable inhibitors for these targets is crucial in putting these targets into practice. Toxicity of some of the inhibitors mentioned is reported, while, traditional Chinese medicine (TCM) may be a good source for screening inhibitors that are less or non-toxic compounds, *e.g.*, an EZH2 inhibitor, davidian, is extracted from TCM *Polygonum capitatum* without toxicity observed in xenograft model[184].

Single effort from one side is far from enough in pancreatic tumour elimination due to its high malignancy and complex tumour microenvironment, multiple targets have to be considered in developing PDAC therapies, therefore, the way of applying these targets and which targets should be applied require further effort.

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REVIEW

Black hairy tongue syndrome

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Abstract

Black hairy tongue (BHT) is a benign medical condition characterized by elongated filiform lingual papillae with typical carpet-like appearance of the dorsum of the tongue. Its prevalence varies geographically, typically ranging from 0.6% to 11.3%. Known predisposing factors include smoking, excessive coffee/black tea consumption, poor oral hygiene, trigeminal neuralgia, general debilitation, xerostomia, and medication use. Clinical presentation varies but is typically asymptomatic, although aesthetic concerns are common. Differential diagnosis includes pseudo-BHT, acanthosis nigricans, oral hairy leukoplakia, pigmented fungiform papillae of the tongue, and congenital melanocytic/melanotic nevi/macules. Clinical diagnosis relies on visual observation, detailed history taking, and occasionally microscopic evaluation. Treatment involves identification and discontinuation of the offending agent, modifications of chronic predisposing factors, patient's re-assurance to the benign nature of the condition, and maintenance of adequate oral hygiene with gentle debridement to promote desquamation. Complications of BHT (burning mouth syndrome, halitosis, nausea, gagging, dysgeusia) typically respond to therapy. Prognosis is excellent with treatment of underlying medical conditions. BHT remains an important medical condition which may result in additional burden on the patient and health care system and requires appropriate prevention, recognition and treatment.

Core tip: Classic descriptors and latest developments in Black Hairy Tongue syndrome. Epidemiology, pathophysiology, etiology, clinical presentation, differential diagnoses, management, complications, and prognosis of Black Hairy Tongue syndrome. First comprehensive review of the syndrome.

Gurvits GE, Tan A. Black hairy tongue syndrome. *World J Gastroenterol* 2014; 20(31): 10845-10850 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i31/10845.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i31.10845

INTRODUCTION

Black hairy tongue (BHT) is an acquired, benign condition characterized by the appearance of abnormally hypertrophied and elongated filiform papillae on the dorsal surface of the tongue. The name is a misnomer and comes from its classical presentation as a superficial black and hairy carpet-like lingual growth (Figure 1). Hairy tongue may also appear brown, yellow, green, blue, or even unpigmented (Figure 2)[1-3]. BHT typically causes aesthetic concerns to the patient and leads to frequent physician visits. It may, however, be rarely associated with gagging, nausea, dysgeusia, xerostomia, burning mouth syndrome and halitosis in some patients[4-6] a constellation of symptoms that, in clinical practice, frequently leads to an evaluation by a gastroenterologist.

Originally described by Amatus Lusitanus in 1557 as hairs on the tongue that would regrow upon being removed, BHT has also been referred to as hyperkeratosis

Figure 1 Classic black hairy tongue.

of the tongue, lingua villosa nigra, nigrites linguae, keratomycosis linguae, and melanotrihia lingua[3,6,7]. Its etiology and pathophysiology have not been fully elucidated and are likely multifactorial. Male sex, older age, smoking, alcohol use, poor oral hygiene, and certain medications place patients at higher risk for developing BHT. Visual inspection is often sufficient for diagnosis. Overall prognosis is excellent as the disease is largely self-limiting and rarely requires procedural intervention.

In this latest review, we discuss the epidemiology, pathophysiology, etiology, clinical presentation, differential diagnoses, management, complications, and finally prognosis of BHT.

EPIDEMIOLOGY

Review of the medical literature shows that BHT is not uncommon. A large cross-sectional study of 5150 Turkish dental outpatients has reported an overall prevalence of 11.3% with increased rates in men (18%) compared to women (6%)[8]. However, a cross-sectional study of 1901 Iranian dental patients only reported a prevalence of 1.2%[9]. BHT occurred in 0.6% of Minnesota school-aged children in contrast to 8.4% patients in a young Finnish population[10-12]. Discordance of the observed rates may stem from difference patient's demographics (age, sex, ethnicity, practices and habits) and interobserver variability in defining lesions in corresponding study populations.

Selected populations are at a higher risk of developing BHT. Patients with oncological disorders, smokers, black tea drinkers, and those with poor oral hygiene are more likely to develop BHT[8]. BHT also shows clear gender and age predilection. Men are about three times more commonly affected than women[8,9,13]. This can be attributed to greater prominence of smoking and higher rates of poor oral hygiene in males. This difference is offset in Finland, where smoking rates have been declining among men and young women are slightly more likely to be affected with BHT[10,14]. In addition, BHT is positively correlated with increasing age with some studies showing a prevalence of nearly 40% in patients over the age of 60[8], though cases have been reported in patients as young as 2-mo-old[2,15]. Although uncommon, in elderly patients,

additional tongue conditions associated with BHT may include fissured tongue (12%) and macroglossia (4%)[8]. Advanced age, poor general condition as well as selected neurological disorders affecting tongue movement and mastication place a patient at a higher risk of developing BHT, largely due to the limited effective friction that results in desquamation of the keratinized layers of the filiform papillae. Finally, globally, there may also be geographic deviations in the prevalence of BHT due to differences in oral hygiene habits and dietary patterns, and variation in oral flora.

ANATOMY AND PATHOPHYSIOLOGY

The tongue is a highly muscular organ located in the oropharynx. It consists of a root, an apex, a curved dorsum, and an inferior surface. The muscles of the tongue are mainly innervated by the hypoglossal nerve, with a small contribution from the pharyngeal plexus[16]. Somatosensory innervation of the tongue is also divided between two nerves. The lingual branch of mandibular division of the trigeminal nerve innervates the anterior two-thirds of the tongue while the glossopharyngeal nerve innervates the posterior third of the tongue. Finally, the lingual artery and its branches supply blood to most of the tongue[16].

BHT typically affects the dorsum of the tongue, which is divided into the oral (presulcal) part and the pharyngeal (postsulcal part) by the V-shaped sulcus terminalis. The dorsal epithelium is lined by non-keratinized stratified squamous epithelium posteriorly and fully keratinized epithelium anteriorly. The dorsal mucosa is directly attached to the underlying muscle with no interposed submucosa. The underlying lamina propria is composed of dense fibrous connective tissue with numerous vessels and nerves supplying papillae. Lingual papillae are protrusions of dorsal mucosa on the presulcal part of the tongue[16].

The four main types of lingual papillae are filiform, fungiform, foliate, and circumvallate papillae. Filiform papillae densely cover most of the presulcal dorsal tongue and are predominately affected in BHT. They are small conical or cylindrical protrusions consisting of a central body surrounded by numerous threadlike cornified projections termed secondary papillae. They function to increase friction between the tongue and food and move particles within oral cavity[16,17].

The pathophysiology of BHT has not been fully elucidated. It is thought to arise from defective desquamation of the dorsal surface of the tongue. This then prevents normal debridement, leading to accumulation of keratinized layers[17]. The resulting hypertrophy and elongation of the filiform papillae appear hairlike superficially. Normally less than 1 mm in length, the elongated papillae can reach a length of 12-18 mm and width of 2 mm[3,4,8,18]. These then secondarily collect fungi, bacteria, and debris[19]. This collection can include residue from tobacco, coffee, tea, and other foods as well as porphyrin-
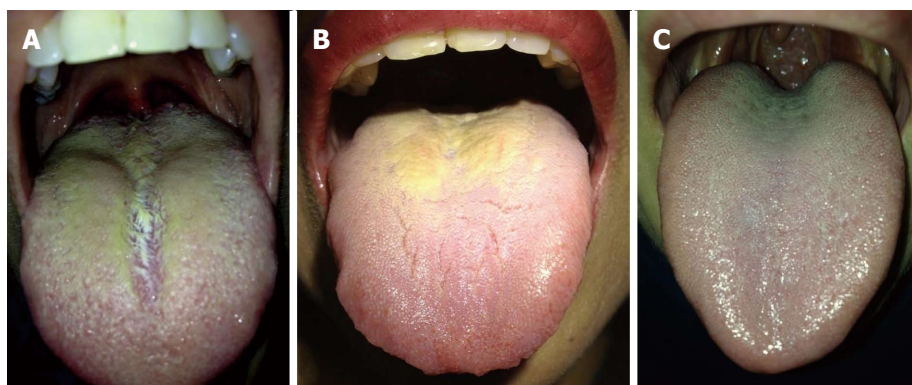
Figure 2 Palette variations of hairy tongue (A and B), Normal tongue (C).

producing chromogenic organisms in the oral flora, which lend the lesion a characteristic hue[7,19]. Using antikeratin probes on BHT epithelium, Manabe *et al*[17] found that the "hairs" are highly elongated cornified spines that result from delayed desquamation of the cells in the central column of filiform papillae and marked retention of secondary papillary cells that expressed hair-type keratins.

ETIOLOGY

The etiology of BHT remains unclear and is likely multifactorial, resulting from combination of local and systemic insults. Various palette appearance of the hairy tongue likely originates in differences in potentially contributing extrinsic (environmental) and intrinsic (chromogenic oral microflora) factors[2]. Although casual smoking poses a slightly increased risk of having BHT compared to non-smokers (15% to 10% in men, 5.5% to 5.2% in women), heavy use of tobacco leads to estimated prevalence of 58% in men and 33% in women[8]. Similar to smoking, heavy black tea consumption lead to increased prevalence of BHT in both male and female patients[8]. Alcohol and intravenous drug use, excessive coffee consumption, poor oral hygiene, general debilitation, and recent radiation therapy to the head and neck region are important risk factors that predispose some patients to develop BHT[6,8,12,20]. Prolonged use of oxidizing mouthwashes containing sodium perborate, sodium peroxide, and hydrogen peroxide has also been associated with the development of BHT[18]. Dietary consumption of herbal tea and sugars may lead to lowering pH on the dorsum of the tongue promoting chromogenic bacterial overgrowth[2]. Most recently, a number of cases of BHT have been reported after allogenic stem-cell transplantation as a cutaneous presentation of graft-versus-host-disease[21]. Finally, prevalence of BHT is increased in malignancies, with one study showing rates as high as 30% in men and 18% in women[22]. Debates on causative relationship between microbial infection and development of BHT date back to 1869 paper by Dr. Raynau, and although previously linked to the presence of various microbial agents, including Candida and Aspergillus species in the oral cavity, microflora found in BHT may be largely coincidental

rather than causative[18,23,24].

Use of systemic and local medications has been commonly implicated in the development of BHT. Antibiotics, including penicillin, aureomycin, erythromycin, doxycycline, and neomycin are most often associated with this disorder[3,4,6,25,26]. However, it should be noted that the cause and effect factor between antibiotics and development of black hairy tongue needs to be further elucidated. Specifically, local or systemic antibiotic use may significantly alter oral flora, thus potentially predisposing the patient to develop BHT. On the other hand, pronounced anatomical alteration in the filiform papillae may predispose the patient to trap foreign material and stimulate local microbial overgrowth that leads to typical color changes seen in patients with this condition. Importantly, earlier studies linking BHT to the use of antibiotics reported local (aerosol or lozenges) oral penicillin use, a type of medication not used in today's medical practice[6]. Additionally, xerostomie agents, including antipsychotics (olanzapine and chlorpromazine) may predispose patients to develop BHT[27,28]. Particular care should be delegated in identifying local inciting factors in the development of BHT, including recent use of new toothpaste or mouthwash[6]. Interestingly, a case of BHT was also reported after four days of erlotinib treatment in a patient with advanced lung cancer, possibly due to an unclear interruption of epidermal grown factor and its receptor in the lingual epithelium[29].

Other diseases and medical conditions associated with BHT include HIV, advanced cancer, and general body illness[6,22]. In addition, BHT has been reported in patients with trigeminal neuralgia. This painful condition, associated with poor oral intake and decreased mastication, is thought to limit tongue movement, resulting in decreased tongue friction with food, palate, and teeth and ultimately hindering normal desquamation of the keratinized filiform papillae, thus leading to the development of BHT[30].

CLINICAL PRESENTATION

A typical patient with BHT is an elderly male smoker on antibiotics or antipsychotics with poor oral hygiene,
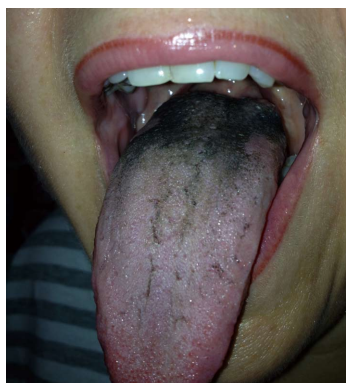
Figure 3 Pseudo black hairy tongue with bismuth salicylate use.

who presents with painless black hair-like lesion on the dorsum of the tongue anterior to the circumvallate papillae. It generally does not occur on the tip or sides of the tongue. Although recognized for its distinctive black color, its hue can range from blackish-brown to yellow-green to unpigmented[7,12]. BHT is generally asymptomatic, though metallic taste, dysgeusia, burning mouth, halitosis, and even gagging have been reported in some patients[31]. Submandibular or cervical lymphadenopathy may occasionally be present in selected cases[2]. Review of systems may be significant for chronic pain, major physical disabilities, psychiatric illnesses, or other debilitating symptoms that preclude the maintenance of proper oral hygiene and normal tongue movement. Other associated clinical conditions include smoking, advanced malignancy, psychiatric conditions, and trigeminal neuralgia. Physical exam is unremarkable aside from the hairy appearing tongue lesion itself. Associated laboratory findings may include positive fungal cultures, HIV test, and blood and urine toxicology studies.

DIFFERENTIAL DIAGNOSIS

Classic BHT presents as a black, hairy-appearing lesion on the dorsum of the tongue (Figure 1). Differential diagnosis includes "pseudo-hairy tongue", oral hairy leukoplakia, pigmented fungiform papillae of the tongue and acanthosis nigricans[31]. "Pseudo-black hairy tongue" (Figure 3) appears as a darkly stained tongue in absence of elongated filiform papillae seen in BHT. Foods, tobacco, and drugs, including antibiotics, antidepressants, and bismuth salicylate, can cause this condition[6,26,32-34]. Oral hairy leukoplakia can be seen in the immunocompromised patients and has a white plaque appearance on the dorsal and ventral surfaces of the tongue, as well as buccal mucosa, and gingiva. Pigmented (due to melanin laden macrophages) fungiform papillae are rare, characterized by isolated hypertrophied lesions primarily on the lateral aspect and apex of the tongue that has a predilection to dark skinned patients. Acanthosis nigricans in the oral cavity manifests as multiple dark and demarcated papillary lesions on the dorsum and lateral region of the tongue with frequent labial involvement and may be as-

sociated with underlying malignancy[31]. Detailed history and physical exam is essential to arriving at the correct diagnosis, with particular emphasis on identifying known etiologic factors. If dubious, biopsy specimens may be required to exclude "mimicking" conditions and confirm the diagnosis. In infants, congenital lingual melanotic macules and congenital melanocytic nevi should be sought for and diligently excluded[2].

CLINICAL DIAGNOSIS

The diagnosis of BHT primarily relies on a visual intra-oral examination. BHT shows a predilection for the dorsal tongue, anterior to the circumvallate papillae and sulcus terminalis. Microscopic examination may be used as an adjunct to diagnosis; demonstrating elongated filiform papillae on the dorsal tongue more than 3 mm in length. Cultures may be considered to rule out superimposed bacterial or fungal infections associated with BHT[35]. Tongue biopsy is supportive but not usually required if the lesion appears characteristic for BHT and responds to mechanical debridement. Careful review of known precipitating factors and recent medication changes is also fundamental in the diagnosis of BHT.

MANAGEMENT

BHT is generally a self-limiting disease and carries a good prognosis. General preventative strategies should be employed and the patient should be educated of this condition as a potential side effect of antibiotic and antipsychotic medications. Care should be taken to promote comprehensive daily oral hygiene. After the diagnosis, a thorough medical history and physical examination are essential in establishing causative relationship to potential environmental triggers and excluding other mimickers of the disease. Proper patient reassurance to the benign nature of BHT is important, both to decrease the level of aesthetic anxiety and to promote appropriate treatment. The goal of therapy is the discontinuation of potential offending agents (including dietary or medicinal causes) and modifying predisposing factors (smoking, black tea consumption, neurological conditions, general debilitation), followed by maintaining good oral hygiene and gentle debridement with a soft toothbrush or tongue scraper to promote desquamation of the hyperkeratotic papillae. Topical application of baking soda or rinsing with diluted hydrogen peroxide solution may help improve desquamation of the keratinized filiform papillae and bleach the color. Lifestyle modifications, including aggressive oral hydration are important and increased dietary consumption of raw fruits and vegetables may help improve this condition by facilitating the roughage on the tongue[27]. Anecdotal use of antimicrobial therapies, topical triamcinolone acetonide, gentian violet, salicylic acid, vitamin B complex, thymol, and topical or oral retinoids (*e.g.*, isotretinoin), as well as keratin olytics (podophyllin), topical 30% urea solution, and trichloroacetic acid have been

reported in the literature, although potential side effects from local irritation and possible systemic absorption are important factors to consider[1,2,18,24,36-38]. Yogurt and probiotic supplementation may be employed with various degree of success. Candida associated glossopyrosis should be treated with antifungal medications. Routine use of proton pump inhibitors is not indicated, although may be of benefit in cases with concomitant severe gastroesophageal reflux disease. Dental evaluation may be indicated in challenging cases, although this is rare. Resistant BHT may require clipping or removal of the papillae by electrodessication or carbon dioxide laser[31].

COMPLICATIONS

Typically, BHT is a self limiting disorder and the development of this condition commonly precipitates only aesthetic concern in affected people. Patients should be reassured about the benign nature of the condition to address anxiety and promote recognition and treatment. In rare instances, patients may report irritation, nausea, and gagging sensation mainly due to the size of unusually elongated papillae. Others may experience a disabling metallic taste, general dysgeusia, and perceived halitosis[18]. Microbial or fungal superinfection is an important consideration in management patients with BHT and proper recognition and treatment may preclude progression to glossopyrosis or burning mouth syndrome.

PROGNOSIS

The long-term outcomes for BHT are excellent as the disease is benign and may even improve spontaneously. Review of the literature shows prompt resolution of this condition within days to few weeks after mechanical debridement and removal of a suspected precipitating agent. Patient education on proper oral hygiene and lifestyle modifications including smoking cessation and alcohol abstinence are vital to preventing reoccurrence. The development of BHT does not typically cause any sequelae. Other co-existing clinical conditions associated with BHT (xerostomia, HIV, cancer, and trigeminal neuralgia) should be sought for and managed appropriately as well to reduce the risk of BHT reappearance.

CONCLUSION

BHT is a relatively common disease that classically manifests as a black and hairy appearing lesion on the dorsum of the tongue arising from abnormally hypertrophied and elongated filiform papillae. Striking as it may appear, this benign condition is usually asymptomatic and its apparent presentation typically triggers only local aesthetic concerns. Rarely, BHT can be accompanied by metallic taste, halitosis, burning mouth, and gagging. Its etiology and pathophysiology continue to be evolving and are undoubtedly multifactorial. Male gender, advanced age, smoking, alcohol abuse, excessive black tea or coffee con-

sumption, HIV, debilitated general condition, and malignancy places patients at a higher risk for developing BHT. Visual inspection and thorough medical history establishes correct diagnosis, although microscopic examination, cultures of tongue swabs, and tongue biopsies may be of additional value in challenging cases. Patients with BHT typically present with indolent self-limited course that responds well to local treatment. Management is primarily focused on mechanical debridement, maintenance of proper oral hygiene, and removal of potential causative agents. Overall clinical prognosis of BHT is excellent.

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REVIEW

Noninvasive biomarkers in non-alcoholic fatty liver disease: Current status and a glimpse of the future

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Abstract
The development of non invasive biomarkers of disease has become a major focus of interest in nonalcoholic fatty liver disease (NAFLD). The large prevalence of the disease and the invasive nature of the investigation means that screening with liver biopsy is impractical. In addition to screening, the differentiation of those with simple steatosis *vs* steatohepatitis and fibrosis is clinically important as the prognosis of each differs. Serum biomarkers may be a combination of simple markers derived from large data sets or direct markers of disease activity. Serum markers of inflammation, apoptosis and oxidative stress in addition to fibrosis have been extensively studied in patients with NAFLD. Other techniques such as transient elastography, magnetic resonance elastography and acoustic radiation force imaging are becoming more established as noninvasive methods of detecting fibrosis in a variety of chronic liver conditions in addition to NAFLD. Newer high throughput methods such as proteomics and glycomics allow the nonhypothesis-driven identification of novel markers and may also potentially contribute to our understanding of the pathogenesis of the condition. This review addresses some of the methodological issues which need to be considered in the search for the ideal biomarker. It is likely that a combination of serum biomarkers and techniques such as transient elastography may provide the optimal diagnostic discrimination however this remains to be proven in large studies.

Key words: Noninvasive biomarkers; Nonalcoholic fatty liver disease; Fibrosis

Core tip: The search for non invasive biomarkers is a major focus of interest in the field of nonalcoholic fatty liver disease (NAFLD). Though the diagnosis of NAFLD is still a histological one, the dramatic rise in prevalence and the spectrum of severity mean that liver biopsy has become impractical for all. Both serum biomarkers of inflammation and fibrosis and assessment of fibrosis using techniques such as transient elastography may have a role to play. Newer techniques (the "omics") may not only lead to novel biomarkers but also allow better understanding of the pathophysiology of the condition.

INTRODUCTION

Ultimately 10% to 28% of nonalcoholic steatohepatitis (NASH) patients develop cirrhosis and hepatocellular carcinoma[1-3]. The criterion standard for diagnosis and assessing progression of disease is liver histology, though this has inherent limitations. Still, the decision "if or when" to perform and repeat a liver biopsy in patients with nonalcoholic fatty liver disease (NAFLD) remains

Serum biomarkers and NAFLD

Caspase-cleaved ck18
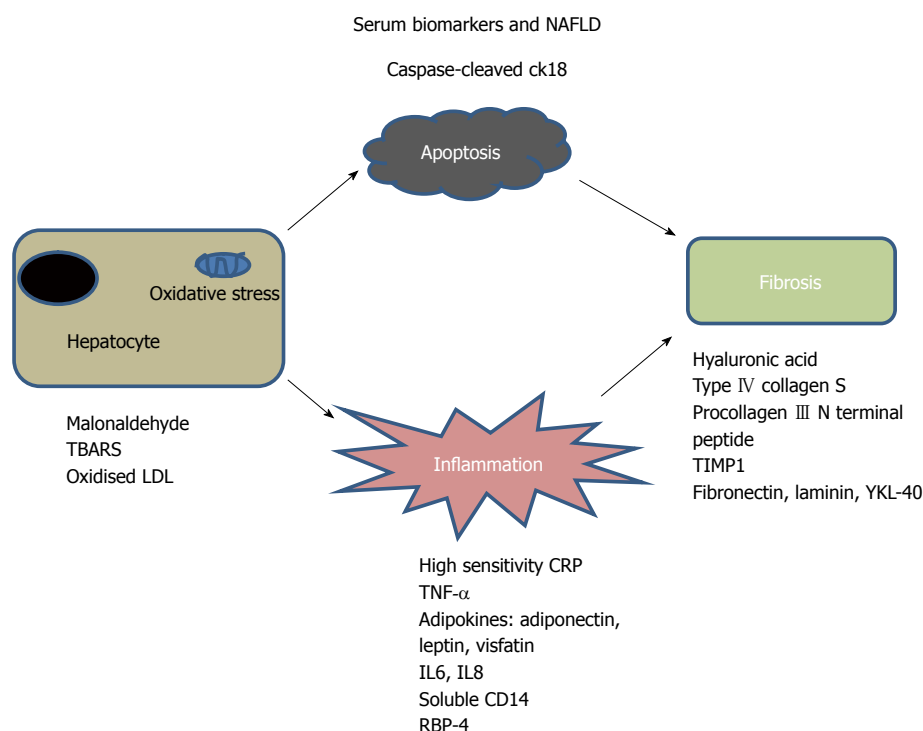
Figure 1 Serum biomarkers of disease activity may measure inflammation, apoptosis, oxidative stress or fibrosis. This is a schematic representation of the patho-physiological processes in nonalcoholic fatty liver disease (NAFLD), markers of which may be demonstrable in serum. LDL: Low density lipoprotein; IL: Interleukin; TBARS: Thiobarbituric acid reactive substances; TIMP1: Tissue inhibitor of MMP 1.

controversial. The prevalence of the condition is such that the resources needed to perform liver biopsy on every patient with NAFLD would be enormous. Liver biopsy often requires admission to hospital and sedation. Risks include bleeding and very rarely death[4]. For the same reason, repeated biopsy is not a suitable tool for regularly monitoring progression of disease or response to treatment. In addition, biopsy samples only 1/50000 of the liver, raising the possibility of sampling error[5].

There has been much focus on the development and validation of noninvasive biomarkers of NAFLD in recent years. There is an urgent need for a less invasive method than biopsy of screening the population, stratifying disease severity and following disease progression. This is particularly relevant in the paediatric population. Many markers of inflammation, hepatocyte apoptosis, fibrosis and oxidative stress are under investigation. The European Association for the Study of the Liver special topic conference on NAFLD called for a renewed focus on noninvasive biomarkers of disease[6]. In common with all biomarkers which are "biological markers of disease presence and progression"[7], important characteristics include; sufficient sensitivity to identify those with disease, specificity to exclude those without disease, cost-effectiveness, ease of use and reproducibility. There are several different approaches to the identification of biomarkers: the first is the use of clinical or biochemical markers that have been derived from large association studies. The second is the use of algorithms including markers of extracellular matrix turnover in the case of fibrosis and inflammation/cell death in the case of inflammatory

change. The third is the non-hypothesis driven new-technology based approach such as microarray techniques, proteomics and glycomics[8,9] (Figure 1).

The pathophysiology and evolution of the particular pathological condition is an important consideration in the development and evaluation of biomarkers. In the case of NAFLD; there are two potential targets. The first is the differentiation of simple steatosis from steatohepatitis. This is important as the prognosis of those with simple steatosis is different from those with NASH[10]. The second issue is the identification of fibrosis stage. This is the main determinant of prognosis and knowing the extent of fibrosis is useful in making treatment decisions, in patient selection for treatment studies and in monitoring progression/regression. Most longitudinal cohort studies in NAFLD have shown that prognosis is determined by stage and rate of progression of fibrosis rather than the presence of necro-inflammation[1,2,11]. Clinical importance lies with being able to differentiate between no/minimal fibrosis (F0/F1), significant fibrosis (F2), severe fibrosis (F3) and cirrhosis (F4).

METHOLOGICAL ASPECTS IN USE OF NONINVASIVE BIOMARKERS OF DISEASE

Important issues to be considered in the design and validation of any noninvasive markers include the inherent limitations of liver biopsy as the criterion standard and the differences in prevalence of different disease stages

Table 1 Biomarkers for the diagnosis of nonalcoholic steatohepatitis (*vs* simple steatosis)

Biomarkers	Study description	Results	Ref.
Simple markers	Adults: 97 obese patients undergoing bariatric surgery, 35 had NASH	Algorithm using AST and presence of T2DM, AUC of 0.82 for prediction of NASH	[55]
	Adults: 80 NAFLD; 39 SS, 41 NASH	Score using age, gender, AST, BMI, Hyaluronic acid, AST: ALT ratio. AUROC for NASH of 0.76	[56]
	Adults: 200 patients undergoing bariatric surgery. 64 had NASH	AUROC for NASH: 0.8 using a score composed of Hypertension, Diabetes, AST > 27, ALT > 27, Sleep apnoea, non-black race	[53]
	Adults: 80 NAFLD; 39 SS, 41 NASH	Score using age, gender, AST, BMI, Hyaluronic acid, AST: ALT ratio. AUROC for NASH of 0.76	[56]
Inflammation	Adults: 57 NASH, 17 SS, 10 controls	AUROC NASH with HOMA-IR and Adiponectin/Leptin ratio: 0.82	[32]
	Adults: 26 NASH, 19 SS, 38 obese, 12 controls	TNF-α, IL8, Age, ALT higher in NAFLD; TNF-α predictor	[27]
	Adults: 20 NAFLD, 30 obese	Insulin resistance, ferritin, glutathione peroxidase, higher in NAFLD than obese	[23]
	Adults: 80 NASH, 29 simple steatosis	Lower Adiponectin, higher TNF-α, higher IR in NASH *vs* controls Lower Adiponectin, higher HOMA-IR in NASH *vs* SS	[30]
	Paediatric: 36 training and 36 validation NAFLD	AUROC for Adiponectin/HOMA-IR as predictors of NASH: 0.79 AUROC for NASH using TNF-α was 0.91, Leptin: 0.8 combined: 0.96	[29]
	Adults: 23 NASH, 21 SS, 18 controls	IL6 and TNF-α, TNFR1 higher in those with NASH *vs* rest TNF-α, CCL2/MCP-1 higher and Adiponectin lower in NASH	[35]
	Adults: 22 SS, 25 NASH, 30 controls		[28]
	Adults: 28 NAFLD, 33 controls, 30 obese	Resistin linked to NAFLD severity, but not adiponectin, leptin or IR	[33]
	Paediatric: 59 NAFLD	RBP-4 levels inverse relationship with NASH	[57]
Algorithms			
NASH test	257 patients (17% NASH) and 383 controls	AUROC 0.79 for NASH. 13 variables: Age, Sex, Weight Height, TG, cholesterol, α2-macroglobulin, ApoA1, Haptoglobin, AST, ALT, γGT, bilirubin	[58]
NASH Diagnostics	Adults: 101 NAFLD, 69 test, (32% NASH) 32 validation	AUROC 0.91 for prediction of NASH. Sensitivity 96%, specificity70% with combination of CK18-M65, CK18-M30, resistin and adiponectin	[44]
NAFIC score	Adult Japanese patients with NAFLD	AUROC for NASH in test group 0.85. AUROC for NASH in validation group 0.78. Variables: Ferritin, fasting insulin, type Ⅳ collagen S	[59]
	177 test group (95 NASH), 442 validation group		
Nice model	Adults: 454 obese, 310 test, 154 validation	Model: AUROC for prediction of NASH 0.88 in test and 0.83 in validation set Algorithm: CK18-M30, ALT, presence of MS	[60]
HAIR	Adults: 105 obese patients undergoing bariatric surgery, including 26 with NASH	Combination of Insulin resistance, Hypertension and ALT gives sensitivity of 80% and specificity of 89% in prediction of NASH	[50]

BMI: Body mass index; AUROC: Area under the curve; NASH: Nonalcoholic steatohepatitis NAFLD: Nonalcoholic fatty liver disease; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TNF-α: Tumor necrosis factor-α; CK: Cytokeratin; IL: Interleukin; HOMA-IR: Homeostasis model assessment-insulin resistance; TG: Triglycerides.

(spectrum bias).

Variations in size of biopsy tissue, number of portal tracts and fragmentation will all influence accuracy of liver biopsy in determining the true stage of fibrosis as described previously[12,13]. In the case of NAFLD the degree of steatosis and inflammation is assessed separately to fibrosis and scoring systems such as the NASH activity score is used to distinguish simple steatosis from steatohepatitis. Both intra and interobserver variability may also significantly affect the score[14]. Thus, the ability of noninvasive biomarkers to differentiate between fibrosis stages is limited by the criterion standard.

Some of these issues in terms of scoring variability may be overcome using techniques such as collagen proportionate area quantification, however the limitations of a short or nonrepresentative biopsy remain.

The ideal outcome measure for any noninvasive biomarker is disease outcome over time, such as has been reported by Parkes *et al*[15]. Long-term outcomes (morbidity/mortality/need for transplantation) are the optimal measures, though are not feasible in shorter term studies.

SERUM BIOMARKERS AND NAFLD

Large adult series have suggested scoring systems using age, BMI, insulin resistance, aspartate aminotransferase/alanine aminotransferase (AST/ALT), platelet count and albumin to differentiate mild from severe disease[16-19] (Table 1). These simple markers are neither sensitive nor specific enough in isolation[20,21]. A growing understanding of the pathophysiology of the disease has allowed the investigation of more specific, mechanism-based biomarkers. These biomarkers focus on the specific pathways involved in the progression of the disease process: hepatocyte apoptosis, oxidative stress, inflammation and fibrosis[8,22,23] (Figure 2).

Markers of inflammation

Generic markers of inflammation such as ferritin and high sensitivity C-reactive protein show an association with NASH[24-26]. Adipokines and other cytokines have been shown to correlate well with presence and severity of the disease[27]. In particular, high serum levels of tumor ne-
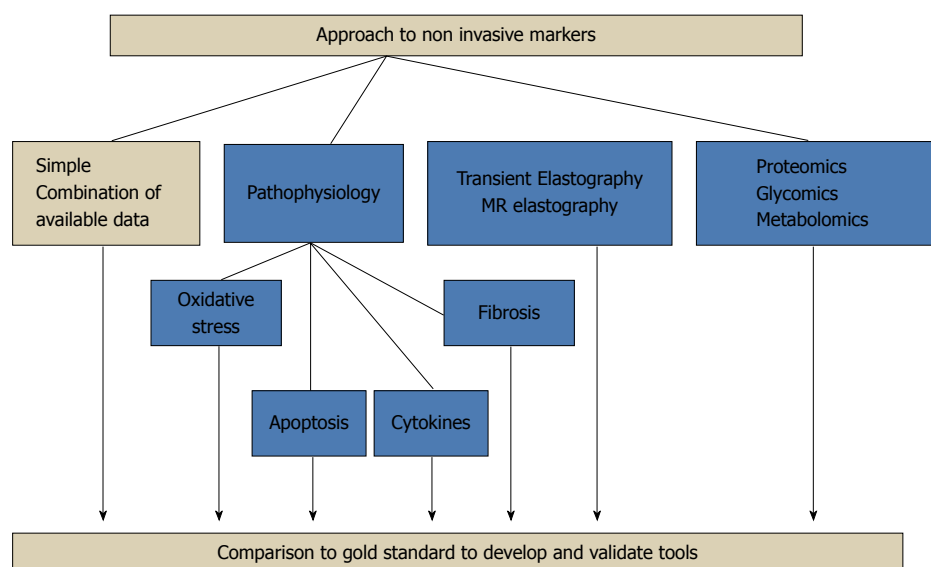
Figure 2 An approach to using noninvasive markers of disease to assess severity of disease in nonalcoholic fatty liver disease.

crosis factor-α (TNF-α) and low levels of adiponectin are associated with greater degree of liver damage[27-30]. Other adipocytokines; visfatin and leptin may be useful predictors of disease though there is inconsistent evidence[28,31]. Lemoine *et al*[32] found that the adiponectin: leptin ratio in combination with homeostasis model assessment insulin resistance index gave an area under the receiver operating characteristic (AUROC) curve of 0.82 for prediction of disease. Resistin was shown by Pagano *et al*[33] to correlate to severity of NASH in a study of 91 patients, but in another study was found to be lower in children with NASH *vs* simple steatosis[34]. Interleukin (IL)6 and IL8 have also been studied and found to have an AUROC of 0.8 for the prediction of NASH[35,36]. The results of circulating levels of adipokines as predictors of disease are inconsistent however and may not be sensitive or specific enough to act as robust biomarkers in isolation.

Markers of cell death

Markers of apoptosis/cell death have been shown to be very useful in differentiating simple steatosis from NASH[37]. The extrinsic (death receptor mediated) and intrinsic (organelle initiated) cell death pathways convene at the mitochondria with permeabilisation of the mitochondrial outer membrane and release of proteins from the mitochondrial inner membrane into the cytosol[38]. Activation of caspase 3 results in cleavage of cytokeratin 18 (CK18) which is a major intermediate filament in hepatocytes. CK18-M30 fragments have recently been shown by a number of studies to correlate well with severity of NASH[39-42]. A two step approach using CK-18 and FGF21 further improves accuracy in diagnosing NASH in one study[43]. CK18-M65 levels (antibodies which recognise uncleaved CK18) are used as biomarkers of total cell death[44] and in one study had equal AUROC to CK18 M30 (0.8) in detecting NASH. Changes in the biomarkers also correlated with histological progression[45].

Markers of oxidative stress

Markers of oxidative stress including lipid peroxidation products, may also be useful biomarkers of disease. However these substances are relatively volatile and not always easily measured in serum. The relative importance of mitochondrial, peroxisomal, CYP450, Nitric oxygen synthetase and myeloperoxidase pathways is not yet known[46]. Malonaldehyde, thiobarbituric acid reactive substances (TBARS) and oxidised low density lipoprotein (LDL) have all been measured as markers of oxidative stress in patients with NASH but with some conflicting results[47,48]. The interaction of molecules such as oxidized LDL and TBARS with stellate cells may be important in promoting fibrosis[49].

Predictive models to distinguish NASH from simple steatosis

A number of predictive models to differentiate either NAFLD from obese controls or simple steatosis from NASH have been developed and validated. Tools include the HAIR score (Hypertension, ALT, insulin resistance) which gives an AUROC of 0.9[50], and the NashTest® (consisting of 13 variables including weight, triglycerides, glucose, α2-macroglobulin and apolipoprotein A) which has an AUROC of 0.79 for differentiation of NASH from simple steatosis[51]. When the NashTest® is combined with the SteatoTest® (10 variables including simple blood tests, age, gender and BMI)[52] and the Fibrotest® into what is known as the Fibromax® panel, the diagnostic accuracy improves further[52]. Campos describes a NASH clinical scoring system using AST, hypertension, presence of type 2 diabetes, ALT, obstructive sleep apnoea and non-black ethnicity. This system has an AUROC of 0.75 for diagnosis of NASH[53]. NASH diagnostics uses a combination of CK 18-M30 and M65 levels with adiponectin and resistin values to give an AUROC of 0.91 in the test and 0.73 in the validation groups. A recent meta-analysis has

evaluated the performance of the NashTest® and ActiTest® for the diagnosis of NASH in 494 obese patients with a prevalence of NASH of 17.2%. The weighted AUROC was significant for the diagnosis of NASH at 0.84 (0.82-0.86, $P < 0.0001$)[54].

NONINVASIVE SERUM BIOMARKERS AND FIBROSIS

It is the severity and rate of progression of fibrosis rather than inflammation per se that determines outcome in the majority of cases[55,56]. The importance of staging disease in the context of fibrosis across liver disease in general is thus manifold. Firstly in the development of treatment decision algorithms; this is particularly relevant in adult viral hepatitis. Secondly functional tests may be even better than biopsy or measurement of hepatic vein pressure gradient in predicting outcome and thus planning appropriate follow up and services[57,58]. Finally the diagnosis of cirrhosis is important so that surveillance for varices and hepatocellular carcinoma may be instigated. These issues are clearly applicable across the spectrum of chronic liver disease, not alone NAFLD[59,60].

NONINVASIVE MARKERS OF FIBROSIS IN NAFLD

Demographics and simple blood tests

Noninvasive markers of fibrosis may consist of simple bedside tests or indices which have been studied in large cohorts of patients with liver disease. These include the AST to platelet ratio index[61], the AST to ALT ratio[62], FIB-4[63] and the Forn's index[64]. These tools have also been validated in the NAFLD population with AUROC between 0.67-0.86 for differentiation of severity of fibrosis[65-67]. Algorithms of simple markers derived from logistic regression analysis of large cohorts with NAFLD are also described. The BAAT score (consisting of BMI, ALT, age and triglyceride levels) has an AUROC of 0.86 for prediction of no fibrosis, 0.75 for F2, 0.92 for F3 and 0.81 for cirrhosis in NAFLD[68]. The BARD score (BMI, AST/ALT ratio, diabetes) was developed in a cohort of 827 patients with NAFLD and was found to be useful in excluding patients without advanced NAFLD[18,69]. Other panels of markers specific for NAFLD include the NAFLD fibrosis score (incorporating presence of diabetes, AST, ALT, BMI, platelets and albumin) giving an AUROC of 0.88 for advanced fibrosis[16]. This was validated by Shah et al[65] with an AUROC for advanced fibrosis of 0.77 and by McPherson et al[66] with an AUROC of 0.84. It has also been validated in Chinese[70] and bariatric surgery cohorts[71]. In a recent meta-analysis the AUROC for the NAFLD fibrosis score was found to be 0.85 with a pooled sensitivity of 90% and specificity of 97%[25].

Fibrometer™ incorporating age, weight, fasting glucose, AST, ALT, ferritin and platelets has been validated in a NAFLD population[67]. The test demonstrates an AUROC of 0.94 for significant fibrosis, 0.9 for severe fibrosis and 0.9 for cirrhosis.

The HAIR algorithm combines presence of systemic hypertension, elevated ALT and insulin resistance and has a sensitivity of 80% and specificity of 89% for NASH in patients undergoing bariatric surgery[50]. The FIB-4 score has an AUROC of 0.8 for advanced fibrosis in 541 patietns with NAFLD[65].

BIOMARKERS OF FIBROGENESIS/ EXTRACELLULAR MATRIX TURNOVER

Other biomarkers measure the degree of extracellular matrix (ECM) turnover. Using such ECM markers is a more direct method of assessing fibrogenic activity, and will tend to measure a dynamic process rather than a static one. Hyaluronic acid is one of the most validated markers of fibrosis in liver disease, synthesised by stellate cells and metabolised by sinusoidal endothelial cells[72,73]. Hyaluronic acid was found to be an accurate marker of fibrosis in NAFLD[74,75].

Combinations of both clinical markers and ECM turnover include the FibroTest®[54,76,77], an algorithm of 13 markers derived from regression analysis including haptoglobin, α2-macroglobulin, apolipoprotein A1, bilirubin, γ-glutamyl transpeptidase, age and gender. It has an AUROC of 0.84 for advanced fibrosis in NAFLD[78].

The European Liver Fibrosis test (ELF)™ combining hyaluronic acid, procollagen Ⅲ N-terminal peptide and TIMP1 was first derived by Rosenberg et al[79] in a cohort of over 1000 patients with chronic liver disease including NAFLD and has since been validated in other NAFLD cohorts with the addition of several simple markers to improve accuracy[80]. Importantly this test has been shown to correlate well with outcome[15].

Table 2 summarises previous studies investigating serum biomarkers of fibrosis in NAFLD[81-84].

NONINVASIVE BIOMARKERS IN PAEDIATRIC LIVER DISEASE

Biomarkers of NAS and fibrosis have also been reported by a few paediatric studies as referenced below. These studies are relatively limited by the size of the cohorts involved and are mostly validation of adult biomarkers.

NASH vs simple steatosis

The following studies report predictors of NAFLD using routine clinical parameters in cohorts of obese children. Sartorio et al[85] reported a multivariate analysis of 267 obese children and found that BMI Z-score, ALT, uric acid, glucose and insulin were useful predictors of NAFLD. Mandato reported insulin resistance, ferritin, C-reactive protein and glutathione peroxidase as good discriminators of those with NAFLD from those without in a cohort of obese children[23]. Neither of these studies used a histological diagnosis of NAFLD.

Table 2 Summarises previous studies investigating serum biomarkers of fibrosis in nonalcoholic fatty liver disease

Biomarkers	Cohort	Results	Ref.
FibroTest®: α2macroglobulin, Apolipoprotein A1, Haptoglobin, γGT, Bilirubin	267 patients	AUROC ⩾ F2 0.8, ⩾ F3 0.88	[81]
NAFLD Fibrosis score: Age, BMI,	733 patients	AUROC ⩾ F3 0.88	[16]
Hyperglycaemia, Platelets, Albumin,	331 patients	AUROC ⩾ F3 0.82	[71]
AST/ALT	162 patients	AUROC ⩾ F3 0.64	[70]
	91 patients	AUROC ⩾ F3 0.89	[80]
	92 patients	AUROC ⩾ F3 0.74	[18]
	235 patients	AUROC ⩾ F2 0.88	[67]
	138 patients	AUROC ⩾ F3 0.68	[69]
	246 patients	AUROC ⩾ F2 0.62, ⩾ F3 0.75	[82]
	588 patients	AUROC ⩾ F3 0.85	[59]
	541 patients	AUROC ⩾ F3 0.77	[65]
	145 patients	AUROC ⩾ F3 0.81	[66]
BARD:	827 patients	AUROC ⩾ F3 0.81	[18]
BMI, AST:ALT ratio, DM	246 patients	AUROC ⩾ F2 0.59, ⩾ F3 0.64	[82]
	138 patients	AUROC ⩾ F3 0.67	[69]
	541 patients	AUROC ⩾ F3 0.7	[65]
	145 patients	AUROC ⩾ F3 0.77	[66]
ELF™	192 patients	AUROC ⩾ F1 0.76, ⩾ F2 0.82, ⩾ F3 0.9	[80]
Hyaluronic acid, P3NP, TIMP1	91 patients (plus simple markers)	AUROC ⩾ F1 0.84, ⩾ F2 0.93, ⩾ F3 0.98	[80]
	121 paediatric patients	AUROC ⩾ F1 0.92, ⩾ F2 0.98, ⩾ F3 0.99	[83]
FibroMeter™:	235 patients	AUROC ⩾ F2 0.94, ⩾ F3 0.94	[67]
APRI	111 patients	AUROC advanced fibrosis 0.85	[84]
	541 patients	AUROC ⩾ F3 0.73	[65]
	145 patients	AUROC ⩾ F3 0.67	[66]
	235 patients	AUROC ⩾ F3 0.87	[67]
AST:ALT ratio	541 patients	AUROC ⩾ F3 0.74	[65]
	145 patients	AUROC ⩾ F3 0.83	[66]
BAAT:	93 patients	AUROC ⩾ F1 0.86, ⩾ F2 0.9	[68]
BMI Age ALT Triglycerides			
FIB-4:	541 patients	AUROC ⩾ F3 0.8	[65]
Age, AST, platelets, ALT	145 patients	AUROC ⩾ F3 0.86	[66]

ELF™: European liver fibrosis score; HA: Hyaluronic acid; PⅢNP: Procollagen Ⅲ amino peptide; TIMP: Tissue inhibitor of MMP; BMI: Body mass index; HOMA-IR: Homeostasis model assessment- insulin resistance; PT: Prothrombin time; AUC/AUROC: Area under the curve; F1-F4: Fibrosis score; TE: Transient elastography; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

Adipocytokines have been investigated in a number of studies. Manco *et al*[29] found that TNF-α and leptin were significantly different in groups of children with NAS ⩾ 5 and NAS < 5[29,86]. Louthan *et al*[87] also used an adipocytokine profile to discriminate steatohepatitis. Other markers include retinal binding protein-4[57] and Fetiun A[88], both of which have been shown to reliably distinguish NASH from simple steatosis/simple obese controls in paediatric studies.

Alisi *et al*[89] investigated both endotoxin and plasminogen activator inhibitor 1 (PAI-1) levels in serum of 40 children with NAFLD and 9 controls and with multivariate analysis found that endotoxin ($P < 0.0001$) and PAI-1 ($P = 0.009$) were significantly higher in patients with a histological score of NAS ⩾ 5. Our group has also reported that the CK18-M30 fragment level is a good discriminator of NASH *vs* simple steatosis[90] following on from the validation of the marker in a large group of adult patients with NAFLD[91].

Noninvasive biomarkers of fibrosis in paediatric NAFLD

As with adult studies, the noninvasive diagnosis of fibrosis (rather than necro-inflammatory change) in NAFLD is considered separately. It is important to acknowledge that the different distribution of fibrosis in paediatric patients may affect the validity of applying measures derived from adult cohorts to this population.

Iacobellis *et al*[19] reported a cohort of 69 children with NAFLD, 60% of whom had fibrosis. They found that BMI was the only significant predictor of fibrosis with multivariable analysis of simple clinical parameters. BMI had an odds ratio of 5.85 for predicting presence of fibrosis. Manco *et al*[92] found waist circumference as a significant predictor of fibrosis in a cohort of 197 children with NAFLD (OR = 2.4, 95%CI: 1.04-5.54). In both these studies the number of children in the F2-F4 groups was small.

Nobili *et al*[93] developed and internally validated the pediatric NAFLD fibrosis index (PNFI) in 136 children with NAFLD. Logistic regression analysis of gender, age, BMI, waist circumference, ALT, AST, γGT, albumin, prothrombin time, glucose, insulin, cholesterol and triglyceridesa were used to develop a predictive model called the paediatric NAFLD with an AUROC for detection of fibrosis was 0.85. Again this study was limited in view of small numbers in fibrosis groups F2-F4.

The ELF™ test was evaluated by Nobili *et al*[83] in 122 children with NAFLD. Simple markers including age, waist circumference and triglycerides were added to improve diagnostic accuracy. Excellent AUROC for any (0.92), significant (0.98) and advanced (0.99) disease were achieved. In this cohort 37 (30%) had no fibrosis, 58 (48%) scored as F1, 9 (7%) as F2, and 8 (6.5%) as F3-F4. Alkhouri *et al*[94] developed this further and validated both the PNFI and ELF™ in a cohort of 111 children with NAFLD (69% with fibrosis). The area under the curve for presence of fibrosis was 0.76 for PNFI, 0.92 for ELF™ and when the two indices were combined: 0.94. The major issue in both studies was the skew towards no or minimal disease, potentially overestimating the accuracy of the test.

NONINVASIVE BIOMARKERS AND IMAGING

Ultrasound, computed tomography and magnetic resonance imaging

Ultrasound (US) has a high sensitivity and specificity for diagnosis of steatosis > 30%, but is not good at detecting fibrosis. Because of the low cost, the absence of radiation exposure and the wide availability, US is often used in screening for NAFLD. The accumulation of fat causes the liver to appear hyperechoic compared with the kidney. This finding is nonspecific and does not differentiate fat from other substances such as glycogen. When compared with histological findings, the sensitivity of US to detect fat infiltration below 30% of the liver is low[95]. Computed tomography (CT) is rarely used for the assessment of NAFLD in children because of its ionizing radiation exposure. Magnetic resonance imaging (MRI) and spectroscopy are the imaging techniques with the greatest accuracy to determine hepatic fat content in studies of both adults and children[96-99]. Aside from liver fat, however, other features of NASH cannot be assessed. Other methods include MR elastography which visualises and measures propagating shear waves and has a high sensitivity (> 85%) and specificity (> 90%) for fibrosis[100]. Cost of this technique may be preclusive however.

For diagnosis of NASH, Iijima *et al*[101] have reported on the use of contrast ultrasound with Levovist with an AUC of 1.0. The decreased accumulation of microbubbles with advancing degree of fibrosis is unique to NAFLD.

Two recent reports have examined the use of acoustic radiation force-based shear stiffness in NAFLD, an ultrasound based investigation which correlates well with the stage of fibrosis in the condition[102,103].

TRANSIENT ELASTOGRAPHY

Transient elastography (Fibroscan®) has been shown to be a useful method for detection of liver fibrosis. This technique uses both ultrasound (5 MHz) and low frequency (50 Hz) elastic waves with a propagation velocity directly related to the stiffness of the liver; *i.e.*, the stiffer

the medium, the faster the wave. The low frequency vibrations are transmitted to the skin by placement of the probe at the intercostal space where a liver biopsy would be performed. A shear wave is induced which propagates into the liver. The wave passes through tissue 2.5-6.5 cm below skin surface, (in those 0 to 7 years a modified probe which can measure 2.5-5.5 cm is used). A pulse-echo acquisition is then used to measure the propagating wave's velocity which is proportional to tissue stiffness represented by the equation for Young's elastic modulus E ($3pv^2$) (p = density, v = shear velocity). Machine based software determines whether each measurement is successful or not. Requirements for accurate evaluation of liver stiffness include an interquartile range of +/- 30% of the median value and ratio of successful measurements to the total no of acquisitions > 60%.

Transient elastography (TE) has been well validated and was the subject of a recent systematic review of 50 studies which concluded that Fibroscan® has excellent diagnostic capability across different liver diseases for cirrhosis[104]. There was some variability for diagnosis of lesser degrees of fibrosis.

TE IN NAFLD

In NAFLD, a number of studies have demonstrated the efficacy of TE in distinguishing severity of fibrosis. In a study of 246 adults with NAFLD, TE had an AUROC of 0.84, 0.93 and 0.95 in distinguishing significant fibrosis, severe fibrosis and cirrhosis respectively[82]. A Japanese study demonstrated similar results[105]. A recent report of 52 children with NAFLD has shown an AUROC of 0.977, 0.992 and 1 for distinguishing any, significant and severe fibrosis[106]. Feasibility and reproducibility of transient elastography is an issue when patients have a BMI > 30[107,108]. An XL probe is now available for better accuracy in this scenario[108,109] demonstrating reliable measurements in 73% using the XL probe *vs* 50% with the S probe[108].

Acoustic radiation force impulse imaging

This is a technology similar to TE in which a region in the liver is targeted and using real-time B-mode ultrasound imaging, the measured shear wave speed is observed at several locations and quantified. Tracking beams are applied adjacent to the push pulse path until the passing shear wave front is detected. The time between the generation of the shear wave and the detection of the peak is used to compute shear wave velocity. Again, this should be proportionate to stiffness of the tissue. This technique has the relative advantage of being able to select an appropriate area for analysis. It is emerging as an effective tool for differentiation of no/mild fibrosis from more severe fibrosis in patients with NAFLD[108,110] with an AUROC of 0.9 in one study[111].

MR ELASTOGRAPHY

MR may be useful in detection of steatosis as above

however the differentiation of patients with advanced disease from those with simple steatosis requires assessment of fibrosis. Similarly to transient elastography, MR elastography (MRE) may be a useful tool in this regard. Kim *et al*[112] report a comparison of MRE to 6 laboratory based models of fibrosis in 142 patients with liver biopsy-confirmed NAFLD. The cut off for advanced fibrosis in this cohort was 4.15 kPa with an AUROC of 0.954, a sensitivity of 0.85 and specificity of 0.929. They found that MRE could potentially be a useful tool but did not meet the sensitivity or specificity of the NAFLD fibrosis score or the FIB-4 score.

Chen *et al*[113] studied 58 patients with NAFLD and found that liver stiffness using a threshold of ≥ 2.74 kPA could differentiate patients with NASH from simple steatosis with a sensitivity of 94% and a sensitivity of 73% (AUROC 0.94).

NONHYPOTHESIS DRIVEN SEARCH FOR NOVEL BIOMARKERS USING NEW TECHNOLOGIES

The use of relatively new, high throughput techniques such as proteomics, glycomics and microarray studies in the derivation of panels of biomarkers associated with a disease may also give an insight into pathophysiology of the condition.

MICROARRAY ANALYSIS

Younossi *et al*[114] found 34 different expression of genes in those with NASH *vs* controls. Four were confirmed using real time reverse transcription PCR. Sreekumar *et al*[115] found 16 genes expressed differently in NASH-associated cirrhosis *vs* other aetiologies; mainly genes which were involved in the anti-oxidant response as well as fat and carbohydrate metabolism. Yoneda *et al*[116] performed a microarray analysis of NASH *vs* simple steatosis and found expression of 27 genes at higher levels in NASH. The upregulated gene sets included those responsible for the platelet derived growth factor, hepatic nuclear factor 3 and the smad4 pathways.

PROTEOMICS

Proteomic studies use pattern recognition with subtraction. Several previous studies have reported different protein peaks in the serum of those with NASH *vs* simple steatosis[117,118]. Two important proteomic studies using liver tissue and serum respectively of adult patients with and without NAFLD revealed an increased expression of lumican, (a keratan sulphate proteoglycan involved in collagen cross-linking and epithelial-mesenchymal transition) in patients with NASH *vs* normal and simple steatosis[119,120]. Yu *et al*[121] used proteomics to demonstrate that higher baseline haemoglobin values were associated with the

development of NAFLD in a prospective study of 6944 subjects.

GLYCOMICS

Glycosylation is the post-translational modification of secreted proteins with carbohydrate moieties conveying structural diversity and with a possible role in protein folding and in cell to cell interaction including migration, solubility and receptor attachment[122,123]. Changes in glycosylation serve as a particularly good marker of liver dysfunction for a number of reasons. Most glycoproteins in serum (aside from IgG) are made in the liver. Thus, the N-glycome profile will reflect any changes in either the liver or B cell function. In addition, both the asialo-glycoprotein receptor and the mannose/*O*-linked beta-*N*-acetylglucosamine receptor in liver are important in clearing aberrantly glycosylated proteins from the serum. In the presence of architectural disarray, these receptors are decreased in number and thus there is a build-up of glycoproteins in serum[124]. With a systems biology approach to the analysis using high-throughput technology, serum *N*-glycomics may prove to be valuable biomarkers of disease.

Previously reported glycomic analysis of liver disease include the development of the GlycoCirrhotest[125], the GlycoFibrotest[126], and the GlycoHCC test[127] which can predict the presence of cirrhosis, fibrosis and hepatocellular carcinoma respectively due to difference in *N*-glycome patterns. Two recent studies have investigated the potential of Glycomics in non-invasive evaluation of NAFLD[128-130].

Glycomics was also demonstrated to have a tole n biomarker discovery in paediatric NAFLD[131].

CONCLUSION

In view of the high prevalence of NAFLD in the population, in both adults and children, and the fact that up to a one third will develop end stage liver disease and/or hepatocellular carcinoma, it is important that we develop noninvasive methods to diagnose and monitor this liver condition. A differentiation needs to be made between those with advance disease/or are at risk of developing advanced disease from those who have simple steatosis and are unlikely to progress. Liver biopsy is not a practical tool for this mass screening though the disease is still defined histologically. Noninvasive biomarkers either in blood or imaging techniques show promise in this context and in many centres are used routinely. It is possible that a combination of blood biomarkers with methods such as transient elastography or acoustic radiation force impulse may yield the highest diagnostic discrimination. New techniques such as proteomics and glycomics may not only allow development of novel markers but also allow us a better insight into the pathophysiology of the condition.

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MINIREVIEWS

Update on imaging of Peutz-Jeghers syndrome

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Abstract

Peutz-Jeghers syndrome (PJS) is a rare, autosomal dominant disease linked to a mutation of the STK 11 gene and is characterized by the development of benign hamartomatous polyps in the gastrointestinal tract in association with a hyperpigmentation on the lips and oral mucosa. Patients affected by PJS have an increased risk of developing gastrointestinal and extra-digestive cancer. Malignancy most commonly occurs in the small-bowel. Extra-intestinal malignancies are mostly breast cancer and gynecological tumors or, to a lesser extent, pancreatic cancer. These polyps are also at risk of acute gastrointestinal bleeding, intussusception and bowel obstruction. Recent guidelines recommend regular small-bowel surveillance to reduce these risks associated with PJS. Small-bowel surveillance allows for the detection of large polyps and the further referral of selected PJS patients for endoscopic enteroscopy or surgery. Video capsule endoscopy, double balloon pushed enteroscopy, multidetector computed tomography and magnetic resonance enteroclysis or enterography, all of which are relatively new techniques, have an important role in the management of patients suffering from PJS. This review illustrates the pathological, clinical and imaging features of small-bowel abnormalities as well as the role and performance of the most recent imaging modalities for the detection and follow-up of PJS patients.

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Core tip: Peutz-Jeghers syndrome is a rare disease characterized by the development of hamartomatous polyps in the gastrointestinal tract. Patients affected by this syndrome have an increased risk of developing gastrointestinal and extradigestive cancers. Regular small-bowel surveillance is necessary to mitigate this increased risk, and recently developed techniques have an important role in the management of Peutz-Jeghers syndrome. This review illustrates the pathological, clinical and imaging features of small-bowel abnormalities as well as the role and performance of the most recent imaging modalities in the detection and monitoring of small-bowel abnormalities in PJS patients.

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INTRODUCTION

Peutz-Jeghers syndrome (PJS) is a rare autosomal dominant, inherited condition linked to a mutation of the STK
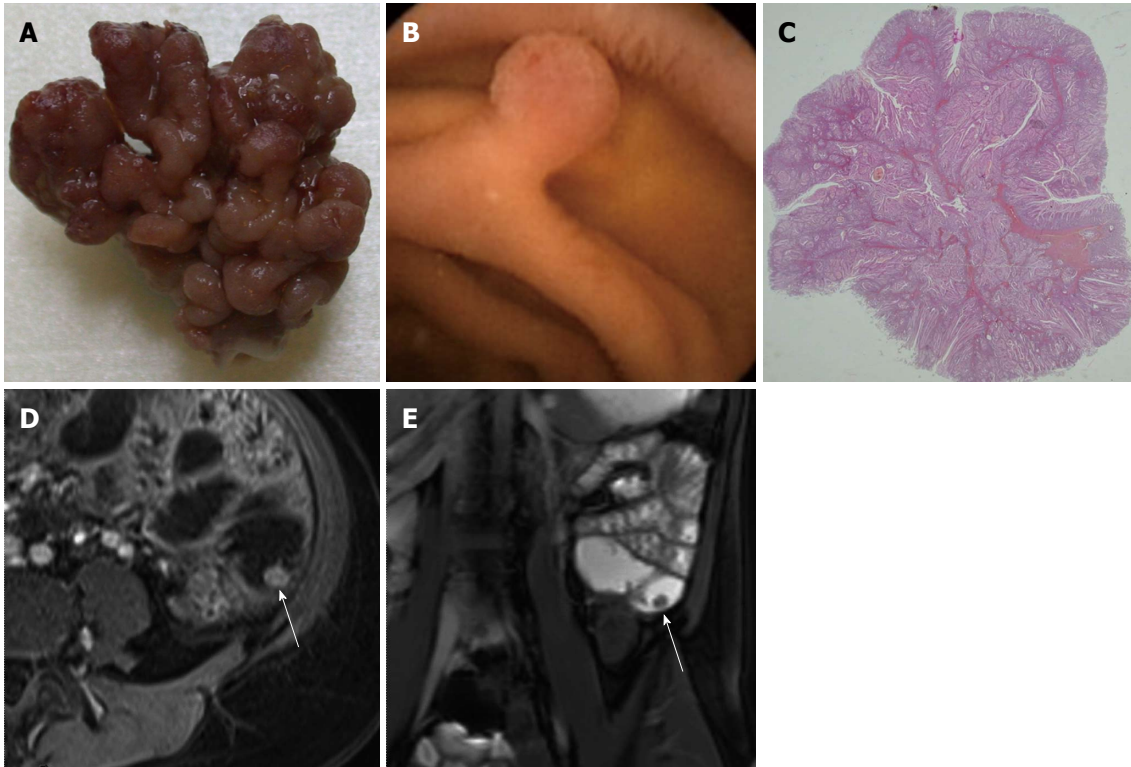
Figure 1 Polyp 1 in a 46-year-old female with known peutz-Jeghers syndrome. A: Macroscopically, the peutz-Jeghers syndrome (PJS) polyp has a coarse lobulated surface; B: The capsule endoscopy image shows that this polyp is pediculate; C: Microscopically, the analysis confirms a benign hamartomatous polyp with a tree-like branching core derived from the muscularis mucosae covered by normal epithelium; D: Fat-suppressed axial T1-weighted-Gadolinium-enhanced VIBE image shows a moderate enhancement of the polyp, with a good characterization of its size and shape (arrow); E: The coronal T2 True Fast Imaging with Steady-state in Precession (FISP) MR image also allows detection of this isolated ileal polyp (arrow).

11 gene and is characterized by a unique type of gastro-intestinal hamartomatous polyp and mucocutaneous pigmentation. Clinical criteria for a definite diagnosis of PJS include the presence of a hamartoma associated with two of the following three signs: family history of PJS, mucocutaneous lentiginosis or polyposis of the small-bowel[1]. The condition is associated with a substantial risk for adenocarcinoma, mainly of the gastrointestinal (GI) tract. Moreover, many patients experience abdominal symptoms before the age of 20 years, in particular because of obstruction and intussusception, which occur in 50% of patients before the age of 20[1-3].

Regular small-bowel surveillance in PJS patients is recommended for two reasons: to reduce polyp-related complications, particularly of intussusceptions, and because of the possible association between PJS and cancer, although there is no data that supports the reduction in risk *via* surveillance[1,4,5]. Surveillance allows for the detection of large polyps and the consequent referral of selected patients for endoscopic enteroscopy or surgery[6,7]. Among the various procedures used for the surveillance of the small-bowel in PJS patients, those that have proven their utility are video capsule endoscopy (VCE), double balloon pushed enteroscopy (DBE), multidetector computed tomography (MDCT) enterography or enteroclysis and magnetic resonance (MR) enterography or enteroclysis.

The aim of this review is to provide an update on imaging presentation of small-bowel abnormalities in PJS as well as the roles and respective performance of the different imaging modalities used in the detection and monitoring of PJS.

PATHOLOGICAL AND CLINICAL FEATURES

Pathological features

Polyps in PJS can develop anywhere within the gastrointestinal tract. The most frequent locations, in order of prevalence, are the jejunum, ileum, duodenum, colon and stomach. PJS polyps are often observed in groups and in up to 20 per segment of the intestinal tract, and PJS polyps have sizes that vary from 1 mm to more than 5 cm. Polyps can also occur elsewhere, such as in the nostrils, lungs, renal pelvis or urinary bladder. Macroscopically, PJS polyps are often pedunculated with a coarse lobulated surface do not have specific endoscopic features[8], and are thus characterized along with the more general group of polyps, hamartomatous polyps (Figure 1).

Microscopically, they are composed of an overgrowth of cells native to the area in which they occur. Their typical histological feature is a tree-like, branched core of muscle derived from the muscularis mucosae covered by normal epithelium with a normal lamina propria[1] (Figure 1).

Small polyps in the bowel may display a phenomenon called "pseudo-invasion," which mimics an invasive carci-

noma. This pseudo-invasion is an epithelial displacement through the muscularis mucosae and can be distinguished from a true invasion by the lack of cytological atypia[3,4].

Clinical features

PJS appears equally in males and females, without any ethnic predominance, at a prevalence of approximately 1/100000[1]. Polyps occur in over 90% of PJS individuals during their lifetime. Many patients will develop gastrointestinal polyps during their childhood or adolescence; the median time to first presentation is around 11-13 years, and half of PJS patients will have experienced symptoms by the age of 20[1,8]. During this time, transient intussusception, small-bowel obstruction and bleeding are common complications. The median age of intussusception is 15 years but with wide variability (range: 3.7-45.4 years)[8]. In Hinds's series dealing with the impact of pediatric screening on the complications of childhood PJS, approximately 30% of the PJS patients required laparotomy before the age of 10 and 68% before the age of 18[9]. Seventy percent of the initial laparotomies were performed urgently for intestinal obstruction[9].

Ninety percent of PJS patients present with a characteristic hyperpigmentation of the skin and mucosa. These dark brown or blue-brown mucocutaneous macules are predominantly located around the lips, mouth, nostrils and the oral mucosa. They often appear during the first decade of life, then fade during adolescence.

PJS patients have an increased risk for gastrointestinal and non-gastrointestinal cancer. A meta-analysis has found that the cumulative risk of developing cancer in PJS patients aged between 15 and 64 years, ranging between 37% and 93%[3]. Malignancy most commonly occurs within the small-bowel, with a median age at diagnosis of 41 years[2]. The risk of colorectal cancer is 3%, 5%, 15% and 39% at the ages of 40, 50, 60 and 70 respectively. Upper gastrointestinal cancers are less common, as the average age for stomach cancer diagnosis is 30[2].

The increased risk of extra-intestinal malignancy is largely due to breast and gynecological cancers in women along with pancreatic cancer, particularly in men[1]. The overall cumulative risk for cancer has been estimated at over 76% in PJS patients and is higher in females than in males, with a risk of breast cancer similar to that of women with BRCA1 or BRCA2 mutations[2,3]. The cumulative breast cancer risk is estimated between 31%-54% at age 60, with a mean diagnosis age of 37. The earliest documented case of breast cancer in PJS was at 19 years of age[4]. The risk of pancreatic cancer is unclear; it varies between 7% and 36% by the age of 60[2,4].

In his study, Giardiello *et al*[1] reported a risk of cervical cancer of 9% by the age of 64, with a mean age at diagnosis of 34 and a risk of 10% for uterine cancer. Giardiello also calculated a 21% lifetime risk of ovarian tumors. Testicular cancer surveillance is also recommended. In a review of the literature, all testicular cancers were Sertoli cell tumors, with a mean age of occurrence of 9 and a range of 3-20 years[1]. The prevalence of thyroid and lung cancers is also slightly increased in PJS but screening for these types of cancer has not been validated[4].

A RATIONALE FOR SURVEILLANCE

The rationale for monitoring polyps of the small-bowel and for treating them early is to avoid mechanical complications and reduce the morbidity conveyed by repeated surgery[4,5,9,10]. Almost 70% of PJS patients have undergone a laparotomy before adulthood[9]. Another objective is to prevent the transformation of these polyps.

Although the mechanism of carcinogenesis in PJS is unknown and remains controversial, the hamartoma-adenoma-carcinoma sequence has been suggested[11]. The risk for developing gastrointestinal cancer in PJS increases progressively with age[2,12]. In theory, the removal of small-bowel polyps would potentially decrease the risk for malignancy by removing precancerous lesions. In the series by Gao *et al*[13], a histopathological analysis of resected polyps showed no malignancy but demonstrated premalignant lesions in up to 18% of the analyzed polyps. Moreover, the risk of intussusception starts early in life, and this complication occurs almost exclusively in the small-bowel.

It is now well acknowledged that polyp size is the most important risk factor for small-bowel intussusception with small-bowel obstruction and that intussusception is generally due to polyps $\geqslant$ 15 mm in diameter[3,14]. Consequently, large polyps (10-15 mm) or symptomatic or rapidly growing polyps should be removed[1,14-16].

Most authors agree that surveillance is needed in PJS patients but there is no consensus as to which organs should be monitored, with what frequency they should be monitored, and at what age surveillance should begin[2,5,9,17,18]. One study suggests that polyps < 10 mm require the monitoring of the small bowel, although those recommendations are based on data of insufficient quality[13]. Nevertheless, the guidelines in Beggs' recent article, produced by a group of European experts, suggest baseline surveillance with esophagogastroduodenoscopy at the age of 8, colonoscopy every 1-2 years after the age of 50, and VCE at 8-10 years of age and then every two to three subsequent years or earlier if any abdominal symptoms are present[4]. For extra-intestinal malignancies, Giardello recommends a monthly breast self-examination starting at the age of 18 years and a semiannual clinical breast examination and annual mammography or MRI starting at the age of 25 years[1]. However, Beggs *et al*[4] suggest that annual MRI/ultrasound surveillance should start at age 25-30 years, substituted with mammography after the age of 50. Routine surveillance for pancreatic cancer has not been proven to be beneficial, but MRI or ultrasonography beginning at the age of 30 years has been proposed[1,3,4]. Beggs also recommends a regular screening consisting of 2-3 yearly cervical smears using liquid based cytology from age 25. The Giardello and Van Lier studies also recommend an annual transvaginal ultrasound and CA-125 screening for ovarian cancer be-

Table 1 Summary of studies throughout the literature dealing with magnetic resonance enterography and the evaluation of polyps in patients with Peutz-Jeghers syndrome

Studies	Design	Number of PJS patients who underwent MR enteroclysis/ enterography	Type of MR-enterography	Comparative method used to evaluate polyps	Results of the study	Impact of MR-enterography in the management of PJS patients
Gupta *et al*[24]	Prospective	19	Enterography per os	VCE	13 MR detected polyps (11-15 mm) with MR *vs* 11 with VCE. 10 MR detected polyps (> 15 mm) *vs* 7 with VCE	MR enterography less prone to missing large polyps
Maccioni *et al*[27]	Retrospective	8	Enterography per os	Enteroscopy/ surgical laparoscopic enteroscopy/surgery	142 MR detected polyps (28 > 15 mm). 187 enteroscopy-detected polyps (30 > 15 mm)	Excellent concordance between MR enterography and enteroscopy for the detection of large polyps (93%)
Caspari *et al*[28]	Prospective	4	Enterography per os	VCE	Equivalent detection rates for polyps > 15 mm with VCE and MR. Better detection of small polyps with VCE. Polyps smaller than 5 mm were exclusively observed with VCE	Identical detection of large polyps with the two methods. Better determination of polyp location and size with MR imaging
Schulmann *et al*[31]	Prospective	4	Enteroclysis	VCE/ push-enteroscopy/ esophagogastroduodenoscopy/surgery	Similar findings of MR enteroclysis compared to VCE in 3 out of 4 patients. Large polyps (up to 30 mm) missed by MR enteroclysis in one patient	VCE is at least equivalent to MR enteroclysis. Small number of patients

MR: Magnetic resonance; PJS: Peutz-Jeghers syndrome; VCE: Videocapsule endoscopy.

ginning at age 25[1,3,4]. Annual testicular examination by testicular ultrasound is recommended in patients where abnormality is detected[4].

These studies emphasize that the surveillance of PJS patients may prolong life expectancy and improve outcomes through the early detection of carcinomas. Gender and age-specific cancer surveillance are important considerations in managing the care of these patients[1-4].

IMAGING MODALITIES FOR DIAGNOSIS AND SURVEILLANCE

In recent years, small-bowel follow-through, which has been the most used diagnostic tool for the assessment of small-bowel polyps, has been uniformly abandoned and replaced by MR imaging, computed tomography (CT) and VCE.

MR Imaging
Details of various relevant studies dealing with the detection of Peutz-Jeghers polyps using MR imaging are listed in Table 1.

MR imaging using dedicated protocols is now being widely used for the evaluation of the small-bowel in a variety of diseases and has been recently proposed as an accurate technique for the detection of small-bowel tumors[19-21].

MRI Protocols: Two fundamentally different MR imaging protocols can be performed for the evaluation of the small-bowel[21]. One of these methods consists of administering an enteral contrast agent *per os* (*i.e.*, MR-enterography), while the other consists of administering the enteral contrast agent directly into the small-bowel using a dedicated naso-jejunal tube (*i.e.*, MR-enteroclysis)[21-23]. The advantages and limitations of each protocol have been discussed in detail elsewhere[21]. For either administration protocol, the use of a biphasic contrast agent is advocated to obtain high contrast between the small-bowel lesion and intraluminal agent. In general, 1.5 to 2 L of enteral contrast agent is needed. MR-enterography and MR-enteroclysis are usually performed in the prone position[20,24-26]. However, remaining intraluminal gas and gas-fluid levels in insufficiently distended small-bowel loops in relation with the orally administered contrast agent may occasionally obscure small polyps. In this regard, Maccioni *et al*[27] suggested that a combined MR-enterography technique using two separate image acquisitions, one in supine position and the other in prone position, helps to increase the number of visible polyps. In their study, MR-enterography detected 142 polyps in eight patients, 114 of which were smaller than 15 mm[27]. The smallest detected polyps with MR-enterography were 3 mm in size. The overall concordance between MR-enterography and endoscopy was 75%, with a higher concordance of 93% for the polyps greater than 15 mm.

Figure 2 Polyp 2 in a 26-year-old female with known peutz-Jeghers syndrome. Axial fat-suppressed T1 weighted-Gadolinium-enhanced axial image shows the enhancement of the rounded PJS polyp (arrow).

Two large polyps were missed, but they were both located in the duodenum. Moving the patients from the supine to the prone position allowed for the detection of additional small (< 15 mm) polyps in four patients and the association of the prone and supine position was significantly more accurate for the detection of smaller intestinal polyps, than supine position only.

MR imaging protocols generally include contrast-enhanced MR sequences, which help detect additional polyps by comparisons with unenhanced MR images[19]. One study has specifically examined the added value of contrast-enhanced T1-weighted MR sequence while performing MR-enterography for small-bowel tumor detection and found that the tumor detection rate is significantly higher on both a per-patient and per-lesion basis after the intravenous administration of gadolinium-chelate[19]. In the study by Gupta *et al*[24] polyp visualization was facilitated by striking enhancement, which was more marked in large polyps. However, polyp enhancement is not a function of tumor size alone, as some small polyps also showed significant enhancement and are better detected by gadolinium-enhanced MR sequences (Figure 2). The actual question that remains unanswered is to what extent the use of gadolinium-chelate may have impact on patient management.

Among unenhanced MR sequences, balanced MR sequences [*e.g.*, Fast Imaging with Steady-state in Precession (FISP), balanced fast field echo, and free induction echo stimulated acquisition] provide the best conspicuity of polyps. Indeed, when using the single-shot-half-Fourier sequence (*i.e.*, half-Fourier acquisition single-shot turbo spin echo or single shot fast spin echo), the presence of flow-void artifacts on images reduces significantly the diagnostic accuracy of MR imaging. This sequence is susceptible to intraluminal motion, and the images might therefore be degraded by low signal intensity, limiting the detection of intraluminal small polyps[20]. Axial planes provide a better identification of the polyps, whereas coronal views allow a better localization of the lesions, which is important for planning the endoscopic or surgical removal of polyps[27] (Figures 1D, E and 3).
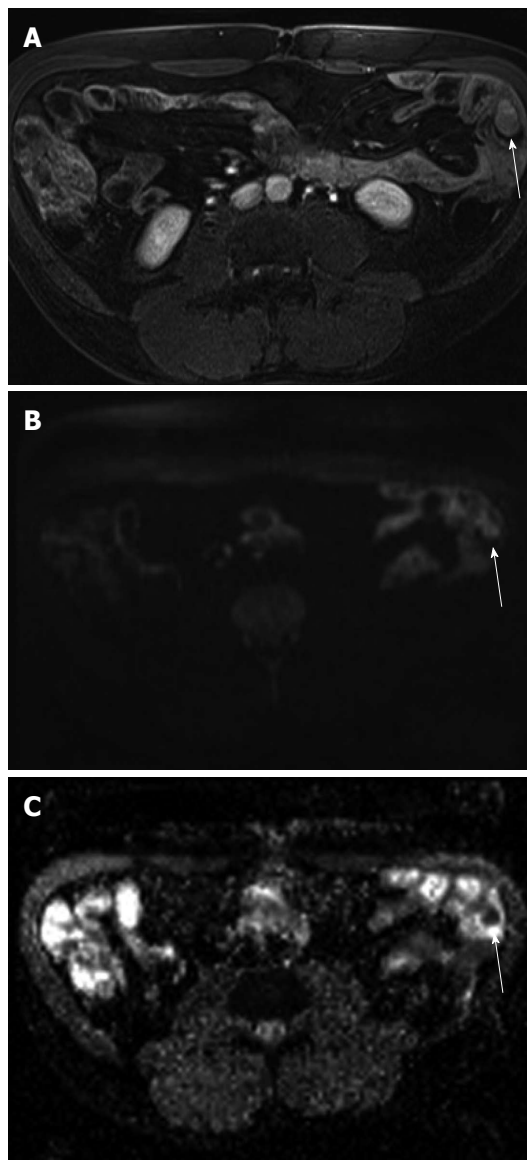
Figure 3 Polyp 3 in a 54-year-old male with known peutz-Jeghers syndrome. A: Axial fat-saturated Vibe gadolinium-enhanced T1-weighted shows a small-bowel slightly enhancing jejunal polyp (arrow); B, C: The corresponding images on a high *b* (*b* = 800) value diffusion-weighted MRI shows a high-signal intensity polypoid lesion inside a jejunal small-bowel loop with a low ADC value compared with the surrounding small-bowel lumen (ADC = 1440 mm^2/s) (arrow).

Caspari *et al*[28] first suggested the use of MR-enterography as an early surveillance tool for PJS patients. Although their study included only 4 patients, they found that MR-enterography was less sensitive than VCE for the detection of small-bowel polyps < 15 mm. However, they concluded that MR-enterography offered the advantage of a more precise assessment of polyp size and localization to a specific small-bowel segment[28].

More recently, two studies have reported satisfactory results for the detection of polyps in PJS patients with MR-enterography in comparison with VCE and balloon pushed enteroscopy or intraoperative enteroscopy[24,27]. In one of these studies, Gupta *et al*[24] prospectively studied a cohort of 19 patients with 41 polyps greater than 10
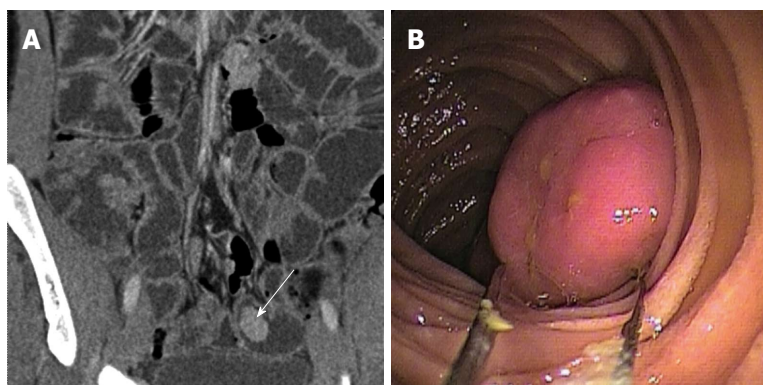
Figure 4 Polyp 4. A: Multidetector computed tomography (MDCT) coronal view reveals a regular small-bowel polyp with homogeneous enhancement (arrow); B: The double balloon endoscopy optimally depicts this large small bowel polyp.

mm, which were detected by either MR-enterography or VCE. There was no significant difference between the two techniques in terms of polyp detection. VCE missed three large polyps (> 15 mm) in three patients that were detected with MR-enterography. However, VCE allowed for the identification of more small polyps (with a size ranging between 6 and 10-mm) than MR-enterography did. The failure of VCE to detect large lesions has been well documented and may be related to luminal debris, a slow frame capture, a limited field of view and rapid transit time, particularly in the proximal small-bowel[29,30]. In another study, Schulmann *et al*[31] reported similar findings using MR enteroclysis in comparison to VCE in three out of four patients, but large polyps (up to 30 mm) were missed in a fourth patient. However, the small number of PJS patients in his study makes these findings less relevant[31]. Moreover, MR enteroclysis requires exposure to ionizing radiation during intubation and is an uncomfortable procedure, making it less appropriate for the surveillance of PJS patients.

The better detection rate of large polyps with MR-enterography has a clinical impact because larger polyps have a greater likelihood to need surgical removal than small polyps, so that their detection has an impact on patient's management[24].

In conclusion, MR-enterography offers a promising alternative to VCE for small-bowel polyps in PJS patients, suggesting the possibility of an effective regular yearly surveillance in patients with this syndrome. Compared to VCE, MR-enterography is also radiation-free, less expensive and more accurate for the identification and localization of large, clinically relevant PJS polyps.

MDCT

MDCT Protocols: MDCT allows for the depiction of small bowel polyps and their complications (*e.g.*, intussusceptions).

Three MDCT protocols can be used. MDCT-enterography and MDCT-enteroclysis are performed for the specific detection of small-bowel tumors and surveillance in patients with PJS, whereas polyp complications, such as intussusception or small-bowel obstruction, are well diagnosed by standard abdominal MDCT.

MDCT-enteroclysis is generally considered to be the optimal imaging technique for SB tumoral detection due to a sensitivity of 92.8% and a specificity of 99.2% for the depiction of small-bowel tumors or, more generally, 97% for the detection of small-bowel diseases[32-34] (Figure 4A, Figure 5A, B and D). MDCT-enteroclysis is generally performed with a standardized protocol. First, a naso-jejunal tube is advanced in the GI tract under fluoroscopic guidance. Room temperature plain water that is used as an enteral contrast material is infused with an electric pump (100-160 mL/min) through the nasojejunal tube. Other enteral contrast agents can be used, such as a water-methylcellulose solution, dilute barium sulfate suspension or commercially produced suspension[21]. A quantity of liquid varying between 1.3 and 1.6 L is needed to obtain optimal small-bowel distension[34]. Continuous water infusion is maintained during scanning. One minute before starting image acquisition, an antispasmodic agent is injected intravenously. Patients are positioned head first, in the supine position. Iodinated contrast agent is injected intravenously before starting the acquisition. MDCT data allows for multiplanar reconstruction and maximum intensity projection (MIP) views. The drawbacks of this technique are the invasiveness of the procedure due to the placement of a naso-jejunal tube and the use of water, which may be contraindicated in patients with renal or cardiac disease because of the potential risk of fluid overflow and radiation exposure[35,36].

On MDCT-enteroclysis, PJS polyps are multiple, regular, often pedunculated lesions of the small-bowel of various size[37,38] (Figure 5D).

Standard MDCT is useful in cases of acute abdominal pain due to small-bowel intussusception in patients with PJS. Intraluminal polyps have a tendency to cause intussusception of the small bowel as peristalsis drags the lesion forward. A pathognomonic bowel-within-bowel pattern suggests intussusception is readily diagnosed by MDCT, appearing either as a target-like or sausage-shaped mass, depending on the orientation with respect to the X-ray beam. The identification of the lead mass is often difficult. Bowel wall edema and the amount of
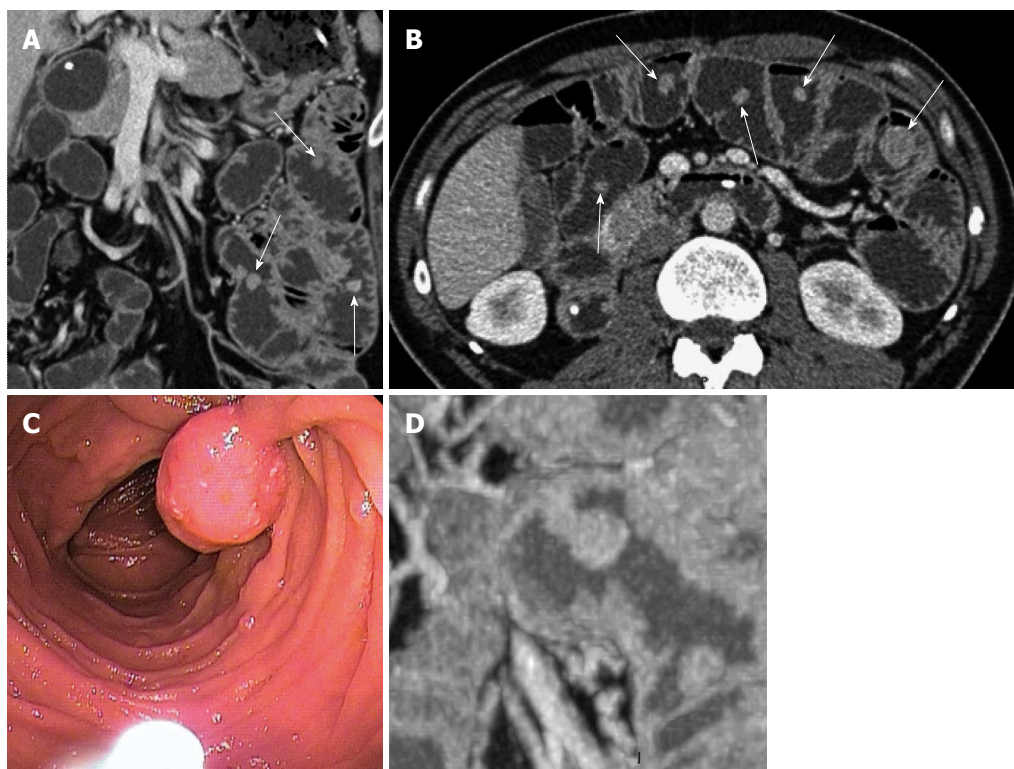
Figure 5 Polyp 5 in a 44-year-old male with a known peutz-Jeghers syndrome. A: Coronal and B: an axial Multidetector computed tomography (MDCT) entero-clysis images reveals multiple, regular polyps with various sizes and shapes (arrows); C: The endoscopic view shows a pediculate polyp; D: A coronal MIP reformat shows the typical pediculate peutz-Jeghers syndrome (PJS) polyp aspect.

Figure 6 Twenty-four-year-old male referred at the emergency department for abdominal pain. The axial Multidetector computed tomography (MDCT) scan shows the bowel-in-bowel appearance with mesenteric fat into the intus-susception (arrows).

invaginated mesenteric fat affect the appearance of the intussusceptions, often leading to an amorphous appear-ance of the mass[39,40] (Figure 6). Multilayered bowel walls, mesenteric fat and vessels of the bowel-within-bowel pattern are also accurately observed on MR imaging. The bowel wall is then thickened with a high signal intensity on T1-weighted and T2-weighted images related to mural hemorrhage and necrosis. Post-gadolinium images show moderate enhancement of the bowel wall due to early bowel wall ischemia[41] (Figure 7).

Endoscopy: Over the last decade, several endoscopic

techniques have been developed, allowing for the visu-alization of almost the entire small-bowel and for thera-peutic interventions, thus obviating the need for a more aggressive surgery in a number of patients[42-44].

Double balloon endoscopy
Since 2001, DBE has been introduced into clinical practice as a modification of the push method and as a method en-abling endoscopic visualization of the entire small-bowel with a success rate of 40%-80%[13,42,43] (Figure 5C and 8B). One balloon is attached to the tip of the endoscope and another is located at the distal end of an overtube. The balloon facilitates the insertion of the endoscope, which can be advanced much further into the small intestine than with push enteroscopy. A main advantage of DBE is that diagnostic and therapeutic interventions can be com-bined in a single procedure, although to date, there is lim-ited data to support such an approach[13]. Before the intro-duction of DBE, the removal of polyps was possible only by intraoperative endoscopy, and in the case of proximal small bowel polyps, surgical resection or push enteroscopy was performed. DBE allows for the endoscopical removal of proximal and distal small-bowel polyps above 10 mm even in young children[44].

However, only one study has compared DBE with other modalities in the detection of small-bowel polyps in PJS patients[14]. Eighteen consecutive patients underwent eighty DBE examinations during 34 sessions. Of these 18 patients, 16 underwent 34 fluoroscopic enteroclysis examinations and 18 patients underwent 38 VCE exami-
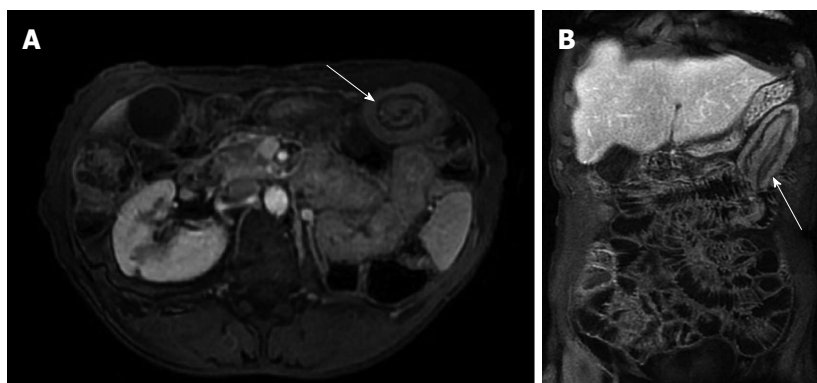
Figure 7 Axial (A) and (B) coronal fat-saturated Vibe gadolinium-enhanced T1-weighted magnetic resonance images illustrate the target-like or sausage shape of the small-bowel intussusception.
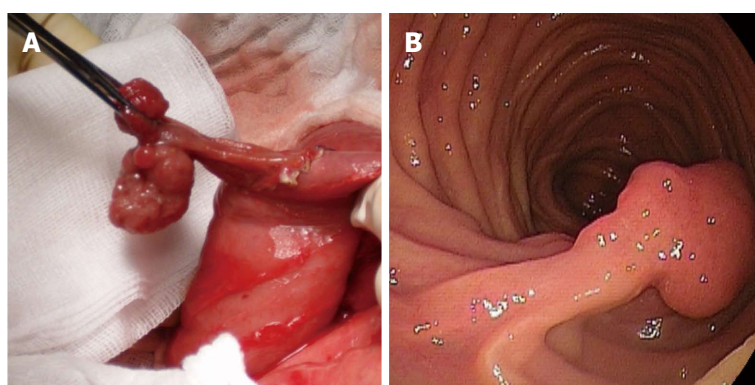
Figure 8 Operative view shows a pediculate polyp that was removed (A) (reprinted with permission from ref.38) after double balloon enteroscopic evaluation (B).

nation. DBE demonstrated more polyps than small bowel follow-through, although both methods found the same number of polyps > 10-mm in diameter. VCE had detection rates similar to those of DBE, regardless of polyp size. Endoscopic resection of 387 polyps, of which 265 were > 10 mm in diameter, was performed during 71 DBE examinations in 16 patients. DBE may outperform the present version of VCE because of the shortcomings of VCE, such as the impossibility of air insufflations, poor maneuverability, interference with total enteroscopy by numerous large polyps and occasional rapid passage of the VCE through the duodenum and the proximal jejunum. Although VCE may outperform DBE in fixed small-bowel loops caused by multiple previous laparotomies, laparoscopic-assisted DBE appears to be promising for PJS patients in the adhesive small-bowel[13,45] (Figure 8).

The rate of complications of DBE ranges between 0 and 6.8%, indicating that DBE and laparoscopic enteroscopy should be limited to the evaluation and endoscopic removal of more advanced intestinal polyps[13,14,46].

Video capsule endoscopy
VCE is a radiation-free diagnostic technique introduced to pediatrics in 2003 and has a few adverse events and complications, although it does not allow for therapeutic procedures[47]. VCE has demonstrated advantages in evaluating obscure and occult gastrointestinal bleeding[21,48].

VCE has been performed on patients with polyposis syndrome in most studies dealing with small bowel tumors detection[49,50] and has shown an improved sensitivity over conventional radiological techniques for polyp surveillance[28,51-54] and a similar detection rate compared to DBE[14].

However, accumulating experience with VCE combined with DBE and MDCT or MR imaging using enterography or enteroclysis techniques has highlighted the potential limitations of VCE technology, particularly in identifying solitary lesions or masses in an otherwise normal small-bowel[29,30,55]. Clinically significant small-bowel lesions can be missed with VCE, even under optimal conditions, especially within the proximal small-bowel[56]. Chong *et al*[56] reported 4 cases of lesions in proximal small-bowel that were detected by DBE after a negative VCE and found that VCE misclassified up to half of patients as having no small-bowel polyps when compared with DBE. Ross *et al*[57] reported 10 patients in whom VCE showed no abnormal findings but who had small-bowel tumors detected by DBE, mostly in the proximal small-bowel. Similarly, Soares *et al*[58] reported that 20% of large small-bowel polyps were missed with VCE in their series. In 7 patients, 26 large polyps were removed; of these 26 polyps, VCE missed five.

It is currently widely acknowledged that the proximal jejunum and duodenum are the most difficult portions
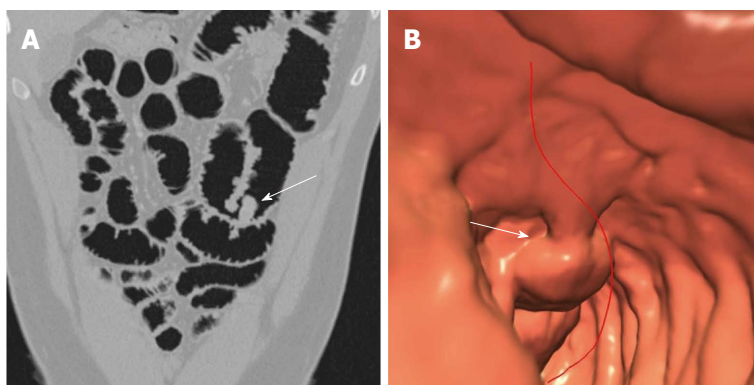
Figure 9 Twenty-eight-year-old male with a known peutz-Jeghers syndrome presenting a single small-bowel polyp. A: Multidetector row computed tomography-virtual endoscopy (MDCT-VE) acquired after gas filling shows a single polyp in the intestinal lumen (arrow); B: Corresponding image of this polyp after MDCT-VE post-processing (arrow).

of the small-bowel to investigate with VCE, most likely because of rapid capsule transit, bubble artifacts and relatively poor luminal distension. In the study by Postgate *et al*[59] a large polyp of 37 mm in the proximal ileum was not detected with VCE but was detected with MR-enterography imaging. Moreover, the major limitations of VCE include a relatively fair interobserver agreement in interpretation and, most importantly, VCE is limited in tumor size evaluation[24,59]. Conversely, MDCT and MR imaging have the undisputed advantage of providing accurate information with respect to lesion size and tumor location. It should also be mentioned that capsule retention is a major complication of VCE and typically requires surgical intervention to remove the retained capsule[49].

FUTURE TRENDS

Spiral Enteroscopy I
Spiral enteroscopy is a relatively new technique for the evaluation of the small-bowel. Spiral (or rotational) enteroscopy, allows for the exploration of more portions of the small-bowel than DBE. This modality allows a therapeutic approach, such as biopsy, hemostasis, or polypectomy. Spiral endoscopy permits the advancement and withdrawal of the enteroscope through the small-bowel with rotating clockwise movements[60]. Morgan *et al*[61], in a prospective, multicenter study, showed that spiral enteroscopy was successful in 93% of patients who were referred for obscure bleeding. The diagnostic and therapeutic yields in this study are as good as previously published data on other deep enteroscopy techniques. Spiral endoscopy is also advantageous in that it involves a shorter examination time (45 min). However, comparative studies of small bowel polyp detection with this technique are warranted.

Virtual Enteroscopy
Recently, virtual enteroscopy has been applied to the evaluation of the small-bowel. Virtual enteroscopy is a promising technique for the detection of small-bowel polyps, although there is a paucity of data in the literature to date. Su *et al*[62] showed that virtual enteroscopy has a high diagnostic accuracy for the detection of small-bowel tumors. In their study, MDCT-virtual enteroscopy identified 30 of 33 cases with proven SB tumors in 125 patients, yielding a sensitivity of 90.9% and a specificity of 96.8% for the detection of small-bowel tumors[62]. The protocol for virtual endoscopy using MDCT includes a liquid dinner the night before the examination and electrolyte solution per os to clean the gastrointestinal tract. Then, the day after, air is introduced into the rectum on the scanning table. The gas in the colon goes into the ileum *via* pressure through the ileocecal valve, filling the small-bowel with gas. Contrast-enhanced scanning is then performed. Post-processing includes three-dimensional rendering, similar to that used in virtual colonoscopy and volume rendering and MIP views[63].

Virtual enteroscopy combined with multiplanar reconstruction is a promising modality for the detection and localization of PJS polyps. Like conventional MDCT, virtual enteroscopy also allows for the analysis of the mesentery. However, to date, only one study has reported the use of virtual enteroscopy in the specific evaluation of a small-bowel tumor; therefore, further studies are needed to clarify the value of this technique for the detection of PJS polyps[62] (Figure 9).

CONCLUSION
MR and MDCT using either enterography or enteroclysis allow for the detection of the majority of polyps in PJS patients. Missed polyps are mostly less than 10 mm in size and are not considered to be clinically significant polyps[29,64,65]. Studies that thoroughly examine the guidelines concerning which examination to perform with respect to its cost-effectiveness and invasiveness are still needed.

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World Journal of Gastroenterology

ORIGINAL ARTICLE

Beneficial effect of butyrate, *Lactobacillus casei* and L-carnitine combination in preference to each in experimental colitis

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Abstract

AIM: To investigate the beneficial effect of the combination of butyrate, *Lactobacillus casei*, and L-carnitine in a rat colitis model.

METHODS: Rats were divided into seven groups. Four groups received oral butyrate, L-carnitine, *Lactobacillus casei* and the combination of three agents for 10 consecutive days. The remaining groups included negative and positive controls and a sham group. Macroscopic, histopathological examinations, and biomarkers such as tumor necrosis factor-alpha (TNF-α) and interlukin-1β (IL-1β), myeloperoxidase (MPO), thiobarbituric acid reactive substances (TBARS), and ferric reduced ability of plasma (FRAP) were determined in the colon.

RESULTS: The combination therapy exhibited a significant beneficial effect in alleviation of colitis compared to controls. Overall changes in reduction of TNF-α (114.66 ± 18.26 *vs* 171.78 ± 9.48 pg/mg protein, $P < 0.05$), IL-1β (24.9 ± 1.07 *vs* 33.06 ± 2.16 pg/mg protein, $P < 0.05$), TBARS (0.2 ± 0.03 *vs* 0.49 ± 0.04 μg/mg protein, $P < 0.01$), MPO (15.32 ± 0.4 *vs* 27.24 ± 3.84 U/mg protein, $P < 0.05$), and elevation of FRAP (23.46 ± 1.2 *vs* 15.02 ± 2.37 μmol/L, $P < 0.05$) support the preference of the combination therapy in comparison to controls. Although the monotherapies were also effective in improvement of colitis markers, the combination therapy was much better in improvement of colon oxidative stress markers including FRAP, TBARS, and MPO.

CONCLUSION: The present combination is a suitable mixture in control of experimental colitis and should be trialed in the clinical setting.

Core tip: Inflammatory bowel disease (IBD) is among the common diseases in the world that have no absolute cure yet. Although corticosteroids, immunosup-

pressants, and aminosalicylates are conventionally used in management of IBD, their side effects reduce patients' compliance. In this paper, we have shown that the combination of butyrate, *Lactobacillus casei*, and, L-carnitine reduces the amount of oxidative stress within the colon and provides significant anti-inflammatory effects. Optimistically, the proposed combination is from components with no serious side effects and is more economical to manufacture.

INTRODUCTION

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease, has an increasing incidence and can be debilitating in the affected patients. Patients with IBD usually suffer from bloody diarrhea, abdominal pain, and also extra-gastrointestinal manifestations such as uveitis, arthritis, skin lesions, and hepatobiliary disease[1-3]. Although the definite etiology of IBD remains debatable, the role of immune dysfunction, particularly over-activity of inflammatory factors including tumor necrosis factor-alpha (TNF-α) and interlukin-1β (IL-1β), and oxidants such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) has been defined along with other factors such as environment, genetics, and intestinal microbes[4,5]. Current protocol of IBD treatment consists of 5-aminosalicylic acid (5-ASA) derivatives, immuno-suppressive agents, corticosteroids, monoclonal antibodies, and some other complementary agents such as herbal medicines. However, increasing complications of conventional medications besides decreased patients compliance have led scientists to focus on the safety alongside efficacy[6-9].

Butyrate, a type of short chain fatty acid, is produced naturally by bacterial fermentation of dietary fibers as a fuel in the colon. Previous reports emphasized its protective ability against oxidative stress and depletion of inflammatory markers including TNF-α and IL-1β through interfering with Ikappa B kinase (IKK) and resulting in down-regulation of nuclear factor-kappa B (NF-κB) which is responsible for generation of pro-inflammatory cytokines[10-13]. In addition, *Lactobacillus casei* (*L. casei*) as a probiotic, exhibits modulatory effects on immune response and oxidative stress *via* IKK or production of anti-oxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). Several studies suggest that manipulation of normal flora content may have beneficial effects in IBD[14-16]. L-carnitine (β hydroxyl-γ trimethyl amino butyrate) plays a significant role in fatty acid β-oxidation, glucose metabolism and general energy control in all types of cells including colonocytes. Production of SOD and inhibition of glutathione (GSH) reduction confirm its anti-oxidant feature and also protective effects against inflammation[17-20]. In summary, recent data revealed that butyrate, L-carnitine, and *L. casei* have noticeable beneficial potential in experimental IBD models alone or in combination with other medicines[21-24]. Therefore, in the present study we evaluated the synergism effect of the combination of these three agents by assessment of inflammatory indicators and pathological markers.

MATERIALS AND METHODS

Chemicals
2,4,6-Trinitrobenzene sulphonic acid (TNBS), butyrate and L-carnitine from Sigma-Aldrich Chemie (Gmbh Munich, Germany), trichloroacetic acid, thiobarbituric acid (TBA), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), N-butanol, hexadecyl tri-methyl ammonium bromide, ethylene diamine tetra acetic acid (EDTA), malondialdehyde, hydrochloric acid (HCL), acetic acid, sodium acetate, hydrogen peroxide (H_2O_2), O-dianisidine hydrochloride, ferric chloride ($FeCl3-6H_2O$), Coomassie reagent, bovine serum albumin (BSA), sodium sulphate (Na_2SO_4), sulphuric acid (H_2SO_4), phosphoric acid (H_3PO_4), potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), sodium carbonate (Na_2CO_3), Na-K-tartarate and cupric sulphate ($CuSO_4-5H_2O$) from Merck (Darmstadt, Germany), whey powder from Shirpooyan-E-Yazd Co. (Tehran, Iran), powder of *L. casei* DN:114001 from Zist-Takhmir Co. (Tehran, Iran) and rat-specific TNF-α and IL-1β Enzyme-Linked ImmunoSorbent Assay (ELISA) kits from (BenderMed Systems GmbH, Austria) were used in this study.

Animals
In this study, male Wistar rats weighing 180-200 g were selected according to regulations of the ethical committee of TUMS approved with code number of 91-03-33-19079. Animals were housed separately in standard polypropylene cages with a wire mesh top, kept under standard conditions including temperature (23 ± 1 ℃), relative humidity (55% ± 10%), and 12/12 h light/dark cycle, and fed a standard pellet diet and water ad libitum.

Experimental design
Animals were divided into seven groups, with seven rats in each group. Colitis was induced by injection of TNBS rectally in all groups except the sham group, which received normal saline. Groups receiving TNBS were divided into control (as an untreated group), positive control (received 1 mg/kg dexamethasone dissolved in water), and treatment groups containing butyrate (1 mL of 0.5% in which 0.5 g butyrate was dissolved in 100 mL PBS), L-carnitine (500 mg/kg in 1 mL), *L. casei* (1 mL of whey culture contains 10^8 Cfu *L. casei*), and combination (0.5 mL butyrate,

0.5 mL L-carnitine, and 1 mL *L. casei*).

Whey culture (10% w/v, 10 g whey powder in 100 mL distilled water) was prepared at 121 ℃ for 20 min. Then, *L. casei* was added to it and incubated at 37 ℃ for 48 h.

The day TNBS was administered was assigned as the first day and all treatments started from the same day. During a 10-d treatment course, the groups were treated by gavage.

Induction of colitis

Prior to induction of colitis, rats were fasted for 36 h. They were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital sodium[25]. Then, 0.3 mL of a mixture, comprising 6 volumes of 5% TNBS plus 4 volumes of 99% ethanol, was instilled through the anus using a rubber cannula (8 cm long) into rats situated on their right side, and then the rats were held in a prone Trendelenburg position to stop the anal leakage of TNBS[26].

Sample preparation

On treatment day 11, animals were sacrificed and colonic tissues were immediately separated. Isolated segments were rinsed with normal saline and then placed in an ice bath throughout the procedure. Colonic tissue was divided into two pieces. The first piece was weighed and kept in 10 mL of formalin 10%, as a fixator for the purpose of histopathological evaluation. The second piece was weighed and homogenized in 10 volumes of ice cold potassium phosphate buffer (50 mmol, pH = 7.4) and then stored at -20 ℃ for 24 h. The sample was then sonicated and centrifuged for 30 min at 3500 *g*, and the supernatant was transferred to a microtube. Then, the sample was kept at -80 ℃ until biomarker analyses.

Macroscopic and microscopic assessments

The following macroscopic scoring system was used to evaluate the severity of colonic damage: 0 - normal appearance with no damage; 1 - localized hyperemia without ulcer; 2 - localized hyperemia with an ulcer; 3 - a linear ulcer with inflammation at one site; 4 - two or more ulcers with damage extending 1-2 cm along the length of the colon; and 5 to 8 - damage extending more than 2 cm along the length of the colon and the score was increased by 1 for each increased cm of involvement.

The microscopic scoring was done by an observer who was blinded to the treated groups. Microscopic scores were determined as follows: 0 - no damage; 1 - focal epithelial edema and necrosis; 2 - disperse swelling and necrosis of the villi; 3 - necrosis with neutrophil infiltration in the submucosa; and 4 - widespread necrosis with massive neutrophil infiltration and hemorrhage.

Myeloperoxidase activity assessment

2.9 mL of 50 mmol/L phosphate buffer containing 0.167 mg/mL O-dianisidine hydrochloride and 0.0005% H_2O_2 was blended with 0.1 mL of the supernatant. The absorbance was measured for 3 min at 460 nm spectrophotometrically (Shimadzu 160A UV-VIS spectrophotometer)

and expressed as unit per mg protein of colon tissue. One unit is equal to the change in absorbance per min at room temperature in the final reaction[27].

Lipid peroxidation assessment

Lipid peroxides as the end products of poly unsaturated fatty acid peroxidation are aldehydes that react with TBA named TBA reactive substance (TBARS) and form a complex which is detected at 532 nm by a double beam spectrophotometer. Concentration of TBARS is recorded as μg/mg protein[28].

Ferric reducing ability of plasma (FRAP) assessment

Ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to bluish ferrous-tripyridyltriazine (Fe^{2+}-TPTZ) with absorption at 593 nm. Values are reported as mmol/L ferric ions reduced to ferrous per mg protein. Details have been described previously[29].

IL-1β and TNF-α assessment

Using ELISA, the quantities of IL-1β and TNF-α (pg/mg protein of tissue) were measured. Amounts of blue complex resulting from conjugation of chromogenic substance with streptavidin-horseradish peroxidise (Streptavidin-HPR) were calculated at both 450 nm (primary wave length) and 620 nm (reference wave length). Details have been described previously[30].

Total protein assessment

Total protein was measured by the Bradford method using BSA as the standard and data are expressed as mg/mL of homogenized tissue at 540 nm[31].

Statistical analysis

One-way analysis of variance followed by Tukey's post-hoc tests was used for multiple comparisons of outcomes, and data are shown as mean ± standard error of the mean (SEM). *P*-values less than 0.05 were considered significant. StatsDirect version 3.0.107 was used for statistical analyses.

RESULTS

Macroscopic and microscopic evaluation of histological impairment

As shown in Table 1 and Figure 1, histopathological examination of the control group which received TNBS showed severe ulcer, diffused necrosis, edema, crypt destruction, and mucosal/submucosal polymorphonuclear (PMN) leukocyte infiltration, which were significantly different ($P < 0.001$) from the sham group that had normal histology and a regular mucosal layer with intact epithelial surface. In the L-carnitine group, crypt destruction and abscess, submucosal inflammation and low PMN infiltration were observed. In the butyrate group, mild infiltration, crypt destruction, edema, and disintegration of crypts were locally observed in some areas. Histopathological parameters in the *L. casei* group were most similar
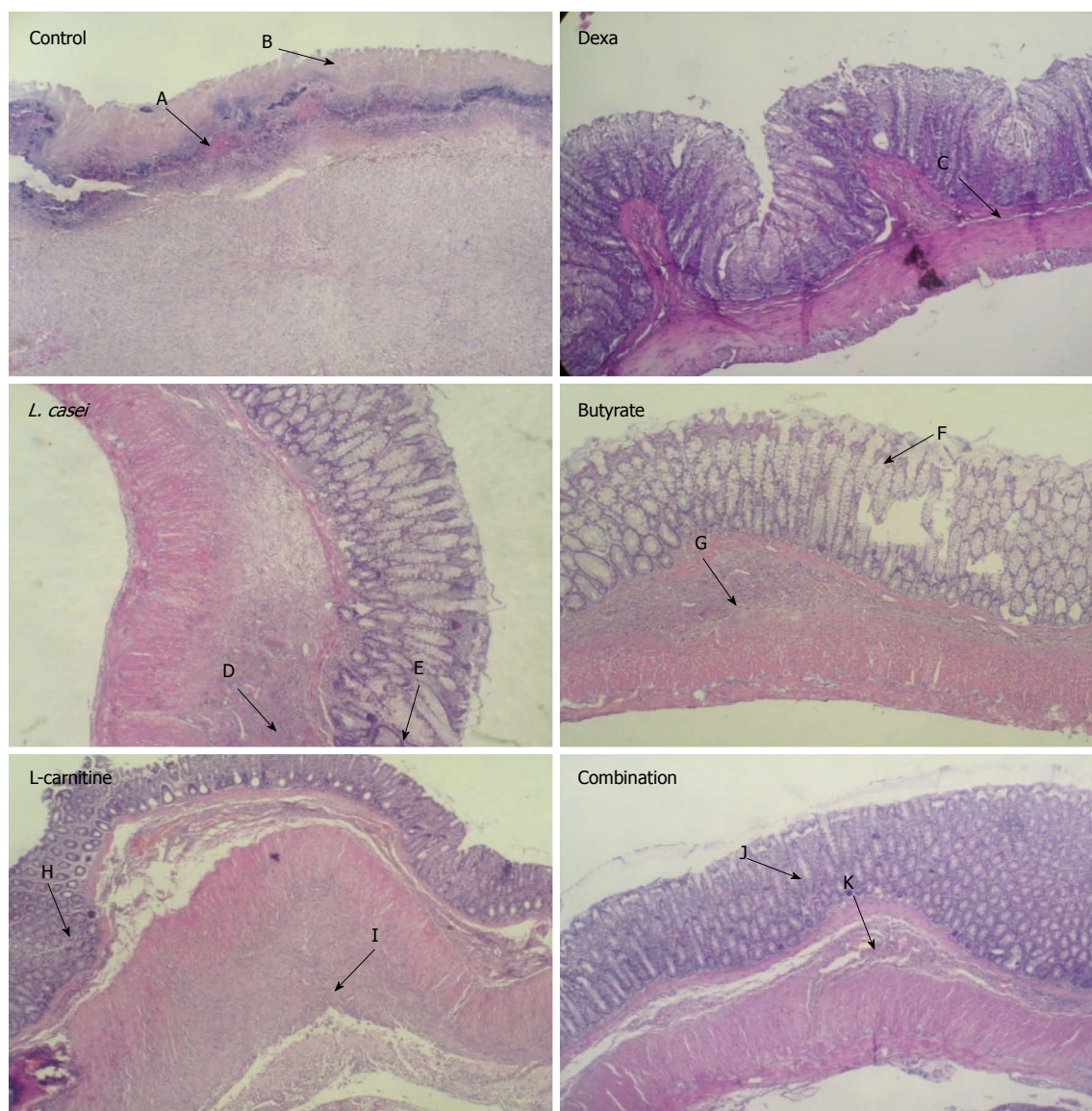
Figure 1 Histological images of colon tissues from control and experimental groups. Microscopic evaluation of Control group showed transmural inflammation and/or diffuse necrosis hemorrhage (A) and severe crypt destruction (B). In histological examination of Dexa group, minimal mucosal inflammation was observed (C). In *L. casei* and Butyrate groups, mild infiltration (D, G) and crypt destruction (E, F) were seen. In L-carnitine group, crypt formation (H), submucosa inflammation and low PMN infiltration (I) were observed. In Combination group, crypt abscess (J) and normal submucosa (K) were evident.

to those in the butyrate group. A significant reduction in microscopic scores was observed in each treatment group in terms of histological symptoms such as inflammation and/or diffuses necrosis hemorrhage and severe crypt destruction in comparison with the control group ($P < 0.001$). In the combination group, crypt abscess and mild inflammation of the submucosa with no PMN infiltration were observed. Although the histological scores decreased in the combination group (Table 1) and histopathological symptoms including PMN infiltration and crypt destruction were more vivid in comparison with monotherapy groups, there was no significant difference between the combination group and monotherapy groups.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity was increased in in-

flamed tissues in the control group in comparison to the sham group ($P < 0.01$). The group of animals receiving monotherapies with butyrate, L-carnitine, and *L. casei* showed a reduction of MPO activity by 40.71%, 39.86%, and 38.95%, respectively, in comparison with controls ($P < 0.05$). Dexamethasone decreased MPO by 60.82% in comparison with control group ($P < 0.01$). Also, in the combination group, there was a significant reduction in MPO activity by 43.75% in comparison with the control group ($P < 0.05$). In the combination, butyrate, and *L. casei* groups, MPO increased by 17.07%, 20.11%, and 20.96%, respectively, in comparison with the dexamethasone group ($P < 0.05$). MPO increased by 21.87% in the L-carnitine group compared with the dexamethasone group ($P < 0.01$). The combination group showed a more reduction of MPO by 4.80% than the L-carnitine group ($P < 0.05$), while there was no significant difference between

Table 1 Extent of colonic damage according to macroscopic and microscopic scores

Groups	Macroscopic score (mean ± SEM); median (min-max)	Microscopic score (mean ± SEM); median (min-max)
Sham	(0.0 + 0.0)	(0.0 + 0.0)
	0 (0.0-0.0)	0 (0.0-0.0)
Control	(6.3 + 0.83)[b]	(3.6 + 0.24)[b]
	6 (4.0-8.0)	4 (2.0-4.0)
Dexamethasone	(1.0 + 0.34)[d]	(1.0 + 0.31)[b,d]
	1 (0.0-2.0)	2 (1.0-3.0)
Combination	(1.00-0.51)[d]	(1.2-0.21)[d]
	1 (0.0-2.0)	2 (1.0-3.0)
Butyrate	(1.6 + 0.5)[d]	(1.4 + 0.24)[d]
	2 (2.0-4.0)	2 (1.0-4.0)
L-carnitine	(2.44 + 0.5)[d]	(1.66 + 0.26)[b,d]
	2 (1.0-4.0)	3 (2.0-4.0)
L. casei	(2.64 + 0.6)[d]	(1.6 + 0.3)[b,d]
	2 (2.0-5.0)	3 (2.0-4.0)

[b]$P < 0.001$ *vs* sham group; [d]$P < 0.001$ *vs* control group.
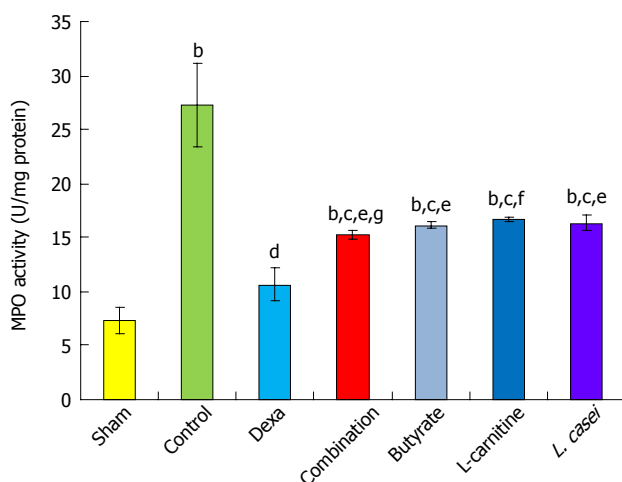
Figure 2 Myeloperoxidase activity in the colon. Values are mean ± SEM. Significantly different from sham group at [a]$P < 0.05$; Significantly different from sham group at [b]$P < 0.01$; Significantly different from sham group at [b]$P < 0.001$; Significantly different from control group at [c]$P < 0.05$; Significantly different from control group at [d]$P < 0.01$; Significantly different from control group at [d]$P < 0.001$; Significantly different from Dexa group at [e]$P < 0.05$; Significantly different from Dexa group at [f]$P < 0.01$; Significantly different from Dexa group at [f]$P < 0.001$; Significantly different from L-Carnitine group at [g]$P < 0.05$; Significantly different from L-Carnitine group at [h]$P < 0.01$; Significantly different from L-Carnitine group at [h]$P < 0.001$.

combination therapy and monotherapies with *L. casei* and butyrate (Figure 2).

TNF-α level

There was an increase in TNF-α level in controls compared to the sham group ($P < 0.001$). In the dexamethasone group, TNF-α was reduced by 54.54% compared with the control group ($P < 0.001$). TNF-α level decreased in the combination, butyrate, and L-carnitine groups by 33.25%, 28.07%, and 19.90%, respectively, in comparison with the control group ($P < 0.05$). Dexamethason was more effective in reduction of TNF-α
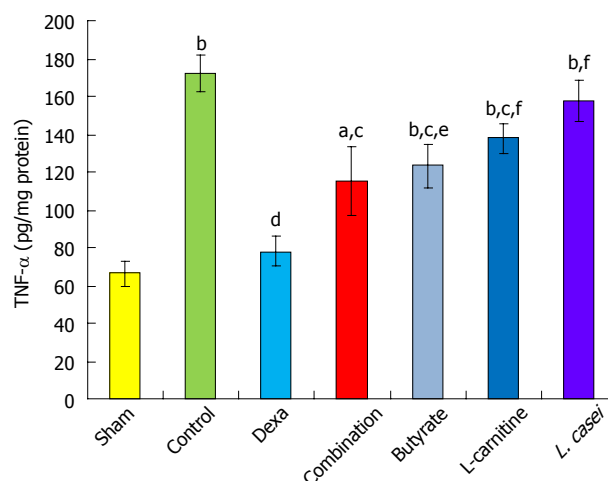
Figure 3 tumor necrosis factor-α level in the colon. Values are mean ± SEM. Significantly different from sham group at [a]$P < 0.05$; Significantly different from sham group at [b]$P < 0.01$; Significantly different from sham group at [b]$P < 0.001$; Significantly different from control group at [c]$P < 0.05$; Significantly different from control group at [d]$P < 0.01$; Significantly different from control group at [d]$P < 0.001$; Significantly different from Dexa group at [e]$P < 0.05$; Significantly different from Dexa group at [f]$P < 0.01$; Significantly different from Dexa group at [f]$P < 0.001$.

than monotherapies with butyrate by 26.46% ($P < 0.05$), L-carnitine by 34.63% and *L. casei* groups by 46.32% ($P < 0.01$). There was no notable difference when comparing combination therapy with single therapies (Figure 3).

IL-1β level

The control group showed a notable elevation in IL-1β in comparison with the sham group ($P < 0.001$). In the dexamethasone, combination, L-carnitine, and *L. casei* groups, IL-1β diminished by 24.98%, 24.68%, 24.13%, and 24.22%, respectively, in comparison with the control group ($P < 0.05$). There was no significant change in the combination group when compared to monotherapies with butyrate, L-carnitine, and *L. casei* (Figure 4).

Anti-oxidant power measured by FRAP

Anti-oxidant power decreased in the control group as compared with the sham group ($P < 0.01$). FRAP value in the dexamethasone group increased by 68.04% ($P < 0.001$) and in the combination group by 56.19% when compared with controls ($P < 0.05$). Monotherapies with butyrate, L-carnitine, and *L. casei* decreased FRAP by 59.92%, 50.73%, and 59.45%, respectively, as compared to the dexamethasone group ($P < 0.01$). The improvements of FRAP by monotherapies with butyrate, L-carnitine, and *L. casei* were 8.12%, 17.31%, and 8.58%, respectively, which were all lower than that in the combination group (56.19%, $P < 0.01$) (Figure 5).

Oxidative stress measured by TBARS

Elevation of TBARS was evident in controls compared with the sham group ($P < 0.001$). Dexamethasone ($P < 0.001$), combination therapy and monotherapies with butyrate, L-carnitine, and *L. casei* ($P < 0.01$) restored
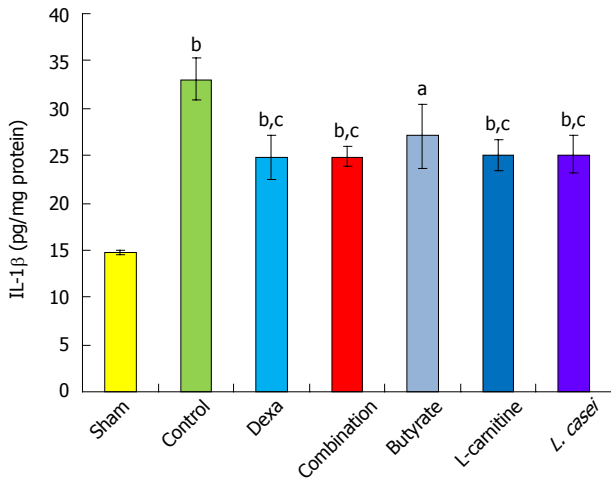
Figure 4 Interlukin-1β level in the colon. Values are mean ± SEM. Significantly different from sham group at [a]$P < 0.05$; Significantly different from sham group at [b]$P < 0.01$; Significantly different from sham group at [b]$P < 0.001$; Significantly different from control group at [c]$P < 0.05$; Significantly different from control group at [d]$P < 0.01$. Significantly different from control group at [d]$P < 0.001$; Significantly different from Dexa group at [e]$P < 0.05$; Significantly different from Dexa group at [f]$P < 0.01$; Significantly different from Dexa group at [f]$P < 0.001$.
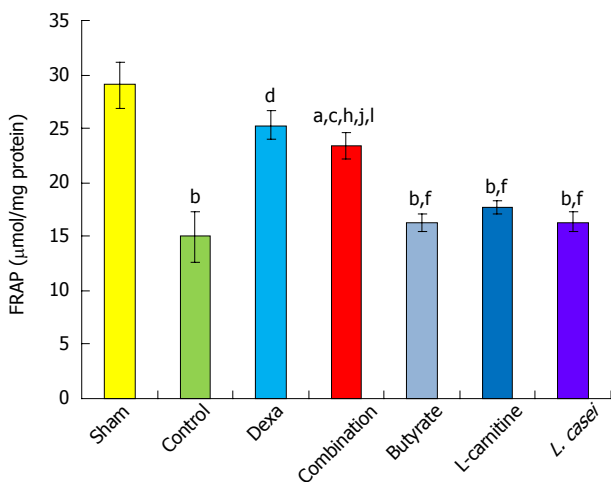
Figure 5 Ferric reduced ability of plasma level in the colon. Values are mean ± SEM. Significantly different from sham group at [a]$P < 0.05$; Significantly different from sham group at [b]$P < 0.01$; Significantly different from sham group at [b]$P < 0.001$; Significantly different from control group at [c]$P < 0.05$; Significantly different from control group at [d]$P < 0.01$; Significantly different from control group at [d]$P < 0.001$; Significantly different from Dexa group at [e]$P < 0.05$; Significantly different from Dexa group at [f]$P < 0.01$; Significantly different from Dexa group at [f]$P < 0.001$; Significantly different from butyrate group at [g]$P < 0.05$; Significantly different from butyrate group at [h]$P < 0.01$; Significantly different from butyrate group at [h]$P < 0.001$; Significantly different from L-carnitine group at [i]$P < 0.05$; Significantly different from L-carnitine group at [j]$P < 0.01$; Significantly different from L-carnitine group at [j]$P < 0.001$; Significantly different from *L. casei* group at [k]$P < 0.05$; Significantly different from *L. casei* group at [l]$P < 0.01$; Significantly different from *L. casei* group at [l]$P < 0.001$.

TBARS by 61.22%, 59.18%, 38.77%, 32.65%, and 38.77%, respectively, in comparison with controls. Reduction of TBARS was significantly lower in the butyrate and L-carnitine groups by 22.44% and 28.57% ($P < 0.001$), respectively, and in the *L. casei* group by 22.44% ($P < 0.01$) in comparison with the dexamethasone group.
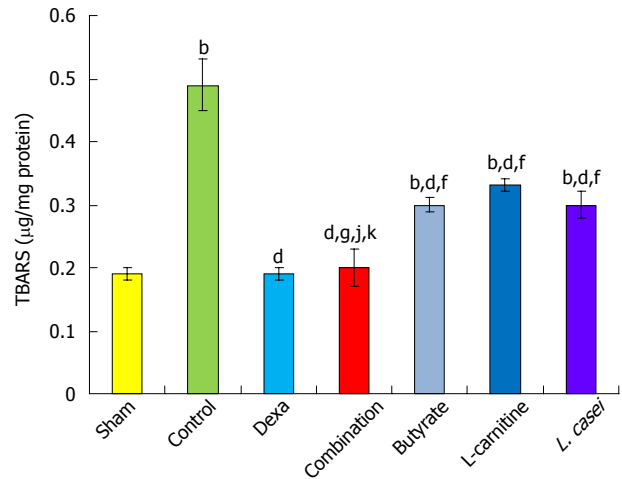
Figure 6 Thiobarbituric acid reactive substances level in the colon. Values are mean ± SEM. Significantly different from sham group at [a]$P < 0.05$; Significantly different from sham group at [b]$P < 0.01$; Significantly different from sham group at [b]$P < 0.001$. Significantly different from control group at [c]$P < 0.05$; Significantly different from control group at [d]$P < 0.01$; Significantly different from control group at [d]$P < 0.001$; Significantly different from Dexa group at [e]$P < 0.05$; Significantly different from Dexa group at [f]$P < 0.01$; Significantly different from Dexa group at [f]$P < 0.001$; Significantly different from butyrate group at [g]$P < 0.05$; Significantly different from butyrate group at [h]$P < 0.01$; Significantly different from butyrate group at [h]$P < 0.001$; Significantly different from L-carnitine group at [i]$P < 0.05$; Significantly different from L-carnitine group at [j]$P < 0.01$; Significantly different from L-carnitine group at [j]$P < 0.001$; Significantly different from *L. casei* group at [k]$P < 0.05$; Significantly different from *L. casei* group at [l]$P < 0.01$; Significantly different from *L. casei* group at [l]$P < 0.001$.

The combination group showed a more decrease in TBARS than the butyrate and *L. casei* groups by 20.40% and 20.40%, respectively ($P < 0.05$). TBARS changed 32.65% in the L-carnitine group, which was significantly lower than that in the combination group (59.18%, $P < 0.01$) (Figure 6).

DISCUSSION

Regarding overall results, the present study demonstrated the priority of combination of butyrate, *L. casei* and L-carnitine in ameliorating the severity of colitis in comparison to monotherapies. Macroscopic features including appearance of isolated tissue and histopathological scores such as presence of edema, necrosis, neutrophil infiltration, and biomarkers including TNF-α, IL-1β, MPO, TBARS, and anti-oxidant power confirmed the beneficial effect of the combination treatment in comparison to controls. Specifically, the combination therapy was much better in reducing colon oxidative stress markers including FRAP, TBARS, and MPO.

Although there are several reports on the positive effects of these three agents in inflammation or oxidative stress explained through various mechanisms[13,14,17], the current study based on an original hypothesis[11] is the first one that confirms synergism between butyrate, *L. casei* and L-carnitine in the immune-based model of colitis. TNBS-induced colitis is believed a preferential model since the colon's barrier is broken by ethanol and
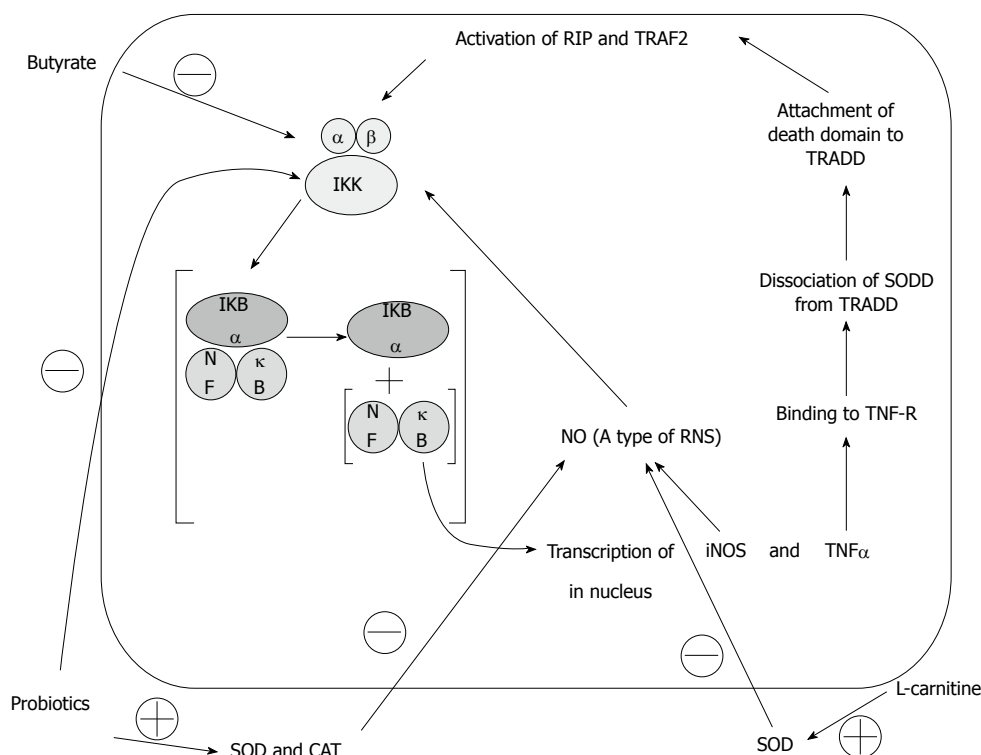
Figure 7 Effects of butyrate, L-carnitine, and probiotics on Ikappa B kinase. Adopted from authors's previous Open Access publication (Moeinian *et al*. Synergistic effect of probiotics, butyrate and L-carnitine in treatment of inflammatory bowel disease (IBD). *J Med Hypotheses Idea* 2013; **7**: 50-53)[11]. SOD: Superoxide dismutase; CAT: Catalase; IKK: IκB kinase; iNOS: Inducible nitric oxide synthase; NO: Nitric oxide; NF-κB: Nuclear factor-kappa B; RIP: Ribosome inactivating protein; ROS: Reactive oxygen species; TNF: Tumor necrosis factor; TNF-R: Tumor necrosis factor receptor; TRADD: Tumor necrosis factor receptor type 1-associated death domain protein; TRAF2: Tumor necrosis factor receptor associated factor 2; SODD: Silencer of death domain.

then delayed-response hypersensitivity reaction by TNBS occurs like that of human IBD[20,32-54]. This similarity is confirmed by the same pattern of changes in examined cytokines between experimental studies and those reported in humans such as accumulation of MPO, TNF-α, IL-1β, ROS, and RNS[4,26,35-38]. Specifically, MPO, a hemo enzyme, is released dramatically from neutrophils in order to eradicate pathogens. It facilitates production of cytotoxic agents like hypochlorite acid (HOCl), the stimulator of NF-κB that is able to induce other inflammatory factors *via* H_2O_2[39,40]. Results indicate that MPO as a neutrophil infiltration indicator and TBARS as an indicator of lipid peroxidation are markedly up-regulated in colon tissues while these are restored by combination therapy. This is most likely due to their radical scavenging properties[3]. As shown schematically in Figure 7, butyrate is able to lessen oxidants through suppressing IKK, which is responsible for dissociation of NF-κB from IKB-α, and then free-NF-κB fortifies oxidants *via* overexpression of inducible nitric oxide synthase gene[4]. In addition, probiotics have the same effect either directly by production of SOD and CAT against oxidants or blocking IKK indirectly[15,16]. Likewise, L-carnitine modifies activity of oxidative stress by elevation of SOD and prevention of decrease in GSH content[17-20], and eventually, the combination treatment exerts well against oxidants with higher efficacy related to synergism of these three agents. In turn, observed changes in the FRAP test support that

idea. Regarding the inflammation, TNF-α, a pro-inflammatory cytokine, is produced mainly by macrophages and activated T-lymphocytes. It is an active component in apoptosis, stimulation of IL-1β secretion, and inflammation, in which re-induction of IKK occurs. This process is mediated through the cascade consisting of binding to TNF receptor (TNF-R) accompanied with dissociation of silencer of death domains from TNF-R type1-associated death domain. Further attachment of TRADD to death domain leads to the activation of the ribosome inactivating protein and TNF-R associated factor 2 which trigger IKK[11,13,41]. In addition, IL-1β has a crucial role in inflammation, proliferation, differentiation, and apoptosis, which is secreted from macrophages, neutrophils, endothelial, and epithelial cells[42]. Our results indicate that in contrast to the control group, abundant formation of IL-1β and TNF-α was restricted in the treatment groups. This is explained by the drop in inflammation either through blocking IKK to activate NF-κB or inability to overexpress inflammatory cytokines. However, obvious differences are evident between monotherapy groups and the combination group in amelioration of inflammation that originates from synergistic effects in the combination therapy.

In summary, a high level of anti-oxidative stress ability, anti-inflammation feature and positive interactions amplify the effects of the combination therapy by overexpression of sodium-coupled mono carboxylate trans-

porter 1 gene, an essential gene for butyrate absorption. Besides, probiotics are able to promote the expression of organic cation transporter number 2, an organic cation transporter that mediates L-carnitine absorption, and thus to enhance β-oxidation of butyrate. This supports the belief that this mixture is more remedial with less complications[11,18,20,33,42-47]. No toxicity following administration of each of them individually or in combination observed in this study certifies the safety of this mixture. Literature shows no severe complications in ordinary patients who take ordinary doses of *L. casei* or L-carnitine[48-50]. However, more investigations are required to clarify the mixture's safety in the clinical setting. According to the results, the combination of these three agents is more effective than each alone in attenuation of the inflammatory process.

ACKNOWLEDGMENTS

COMMENTS

Background
Inflammatory bowel disease (IBD) is divided into two major and idiopathic conditions, ulcerative colitis and Crohn's disease. Current choice in control of IBD is administration of aminosalicylates, corticosteroids, and immunosuppressants that are associated with side effects. Due to side effects of current conventional therapies, scientists have tried to discover more potent and safer alternatives. The authors intended to demonstrate that the combination of butyrate, L-carnitine, and *L. casei* may be an effective and safe mixture in amelioration of colitis.

Research frontiers
Several studies focused on the major role of nuclear factor-kappa B (NF-κB) signaling which results in overproduction of inflammatory mediators and oxidants in the progress of inflammation. In this study, the authors evaluated the capacity of the combination therapy in healing the colitis and improvement of inflammatory cytokines.

Innovations and breakthroughs
The present study indicated that the combination therapy is able to heal colitis and down-regulate the production of inflammatory cytokines. Inhibition of the NF-κB pathway either by suppression of IKK or production of enzymes against free radicals is the main mechanism of action of this mixture.

Applications
Safety and effectiveness besides positive interactions of these three agents confirm that this combination can be more helpful in healing IBD and improving the patients' quality of life.

Peer review
The main hypothesis of the paper for management of IBD is valuable.

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ORIGINAL ARTICLE

Oxytocin decreases colonic motility of cold water stressed rats *via* oxytocin receptors

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Abstract

AIM: To investigate whether cold water intake into the stomach affects colonic motility and the involvement of the oxytocin-oxytocin receptor pathway in rats.

METHODS: Female Sprague Dawley rats were used and some of them were ovariectomized. The rats were subjected to gastric instillation with cold (0-4 ℃, cold group) or room temperature (20-25 ℃, control group) saline for 14 consecutive days. Colon transit was determined with a bead inserted into the colon. Colonic longitudinal muscle strips were prepared to investigate the response to oxytocin *in vitro*. Plasma concentration of oxytocin was detected by ELISA. Oxytocin receptor expression was investigated by Western blot analysis. Immunohistochemistry was used to locate oxytocin receptors.

RESULTS: Colon transit was slower in the cold group than in the control group ($P < 0.05$). Colonic smooth muscle contractile response to oxytocin decreased, and the inhibitory effect of oxytocin on muscle contractility was enhanced by cold water intake (0.69 ± 0.08 *vs* 0.88 ± 0.16, $P < 0.05$). Atosiban and tetrodotoxin inhibited the effect of oxytocin on colonic motility. Oxytocin receptors were located in the myenteric plexus, and their expression was up-regulated in the cold group ($P < 0.05$). Cold water intake increased blood concentration of oxytocin, but this effect was attenuated in ovariectomized rats (286.99 ± 83.72 pg/mL *vs* 100.56 ± 92.71 pg/mL, $P < 0.05$). However, in ovariectomized rats, estradiol treatment increased blood oxytocin, and the response of colonic muscle strips to oxytocin was attenuated.

CONCLUSION: Cold water intake inhibits colonic motility partially through oxytocin-oxytocin receptor signaling in the myenteric nervous system pathway, which is estrogen dependent.

Key words: Intragastric cold water stress; Colonic motility; Estradiol; Oxytocin; Oxytocin receptor; Irritable bowel syndrome

Core tip: Colon transit was decreased and oxytocin-induced inhibition of colonic contraction was enhanced in rats with cold water intake. Atosiban and tetrodotoxin inhibited the effect of oxytocin on colonic motility. Cold water intake increased blood concentration of oxytocin and expression of oxytocin receptors in colon. Estradiol regulated blood concentration of oxytocin and oxytocin-induced colonic contraction. The results sug-

gested that cold water intake inhibited colonic motility through oxytocin-oxytocin receptor signaling in the myenteric nervous system pathway, which is estrogen dependent. The estradiol-oxytocin-oxytocin receptor-colonic contractile pathway might be a new therapeutic target for irritable bowel syndrome in females.

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional bowel disorder in which abdominal pain or discomfort is associated with defecation and features of disordered bowel habit[1]. Throughout the world, about 4%-20% of adults and adolescents have symptoms consistent with IBS, and a female predominance has been found by most studies[2-6]. Altered colonic motility, visceral hyperalgesia, disturbance of brain-gut interaction, abnormal central processing, autonomic and hormonal events, genetic and environmental factors, postinfectious sequels, and psychosocial disturbance are variably involved in the development of IBS symptoms[7-9]. Consuming cold meals and cold drinks is common among some people, especially in the industrialized countries. Zuo *et al*[10,11] found that cold water intake increased plasma 5-HT concentrations and lowered visceral perception thresholds in subjects with IBS. Previous studies also found that cold meal intake affected gastric myoelectrical and contractile activities[12,13]. Healthy women with excessive exposure to cold stress show an abnormal intestinal response to incoming stimuli[14]. Cold water intake might alter colonic motility and be involved in IBS in females.

Oxytocin (OT) is a neuropeptide synthesized by magnocellular neurons in the paraventricular and supraoptic nuclei of the hypothalamus. It is best known as a critical social and reproduction hormone[15-17]. OT effects are more generally associated with trait effects related to social or emotional functions, particularly in females[18]. The oxytocinergic system selectively influences the functional response of females, and as such may be involved in normal and pathologic states that are more common among females. Our previous studies showed that OT decreased the contraction of colon, which is ovarian steroid-dependent[19,20]. In response to a variety of stimuli such as suckling[21], parturition[22], or certain kinds of stress[23-26], the processed OT is released from the posterior pituitary into the systemic circulation. Chronic hyperosmotic stress (drinking water with 2% NaCl solution for 7 d) increased the secretion of OT[27]. Whether intragastric cold stress affects release of OT and then affects colonic contrac-

tion in females is still unknown.

OT receptors are expressed in the gastrointestinal tract[28]. OT has been reported to affect colonic smooth muscle contraction *via* OT receptors[19,20]. OT receptors are found in the gastric smooth muscle[29], the enteric nervous system (ENS) or intestinal epithelium[30-32]. Intragastric cold stress might also affect expression of OT receptors in the colon.

Ovarian hormones are found to regulate the expression of OT receptors in various tissues and the tissue response to OT[33-36]. Estradiol (E_2) is known to regulate OT expression in the hypothalamus[37-40].

In the present study, we hypothesized that intragastric cold stress stimulates the secretion of OT and upregulates the expression of OT receptors to affect colonic motility in female rats and that this effect is exerted *via* OT receptors in colon, and is estradiol dependent.

MATERIALS AND METHODS

Animals

Female Sprague Dawley rats were housed in a temperature (22 ℃)-controlled environment. The use and treatment of animals were approved by the Animal Care and Use Committee of Tongji University (approval no: 2009-0022). All animals were cared for in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals, published by the National Science Council, China. The experiments conform to the Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes. The experiments were done two days after the last intragastric cold stress procedure. The rats were fasted overnight with water *ad libitum* before the experiment. Pain or discomfort of the rats was minimized during the experiments.

Intragastric cold stress protocol

The rats were 6-8-week-old and weighed 180 ± 10 g at the beginning of the treatment. Animals were subjected once daily at 8 am to gastric instillation with cold (0-4 ℃) physiological saline as the cold group or room temperature (20-25 ℃) physiological saline (1 mL) as the control group, for 14 consecutive days. Room temperature regular drinking water and food were offered to rats in both groups all the time.

Ovariectomy

Some of the rats were ovariectomized. Ovariectomy was performed quickly under light ether anesthesia. The ovaries were picked out by forceps through a 1-cm incision made over both flanks. A ligature was placed below the ovary and the ovary was removed, then the incisions of the muscle and skin were sewed up by aseptic suture line, and finally the wounds were disinfected by Iodophors.

Measurement of colonic transit

The rats were lightly anesthetized with sevoflurane. A

single 3-3.5 mm glass bead was lubricated with vaseline. The anus was opened with a lubricated glass rod. After the bead was put into the anus, it was inserted into the colon (3 cm proximal to the anus) quickly with the glass rod. The evacuation time was monitored after consciousness was regained.

Preparation of isolated colonic smooth muscle strips
A segment of the colon of approximately 4 cm was collected and put in Krebs solution (composed of NaCl 118.5 mmol/L, KCl 4.8 mmol/L, KH_2PO_4 1.2 mmol/L, $MgSO_4$ 1.2 mmol/L, $CaCl_2$ 1.9 mmol/L, $NaHCO_3$ 25.0 mmol/L and glucose 10.1 mmol/L). The segment was opened along the mesenteric border and pinned mucosa side up. The mucosa was removed by sharp dissection and the muscle strip (2 mm wide and 8 mm long) was cut along the longitudinal axis. Silk thread was attached to both ends of the muscle strips, and the strips were mounted in 5-mL organ baths. The organ baths contained aerated (5% CO_2, 95% O_2) Krebs solution which was maintained at 37 ℃. Strips were adjusted in length to an initial tension of 1 g, and were allowed to stabilize for 60 min before experimental procedures were initiated. Isometric tension was measured using external force transducers (JH-2B, Beijing, China). Force signals were amplified with a SMUP-PC amplifier (Fudan University, Shanghai, China), and recorded using an MFlab system.

Western blot
Samples of colon stored at -80 ℃ were homogenized for protein analysis. The homogenates were centrifuged at 2000 rpm for 10 min at 4 ℃, and the protein content of the supernatants was evaluated using Protein Quantitative Analysis Kit (k3001-BCA; Shenergy Biocolor, Shanghai, China). Supernatants containing 100 μg protein were diluted in reducing × 2 sample buffer and loaded into 12% SDS-PAGE. After separation by SDS-PAGE, proteins were transferred to nitrocellulose membranes. Membranes were blocked for 3 h at room temperature in blocking buffer (5% nonfat dry milk and TTBS), washed in TTBS (0.1% Tween 20, 50 mmol/L Tris, and 150 mmol/L NaCl) , and incubated overnight with anti-rat oxytocin receptor IgG (1:400, sc 8102; Santa Cruz Biotechnology, CA, United States), followed by peroxidase-conjugated secondary antibodies (1:20000). Finally, immunoreactive proteins were revealed using twin plate Color Scanner (T1200; AGFA, Shenzhen, China).

Immunohistochemistry
Immediately after the animals were anesthetized with sevoflurane, a segment of distal colon was removed and soaked in 4% paraformaldehyde for 12 h. The fixed tissue was rinsed for 100 min and was dehydrated, cleared and mounted in wax. The tissue was cut into 4-μm sections, and stained by a two-step method. Activity of endogenous peroxidase was blocked with 3% hydrogen peroxide. After three rinses in PBS, 10% normal rabbit serum was applied for 15 min, and then the sections were incubated with primary goat anti-oxytocin receptor antibody (Santa Cruz, diluted 1:100 in PBS) overnight in a humid chamber at 4 ℃. After the sections were washed, they were incubated with polymer peroxidase-anti-goat serum (ZSGB-BIO, Beijing, China) for 30 min at room temperature. After several rinses, peroxidase was revealed using a 3, 3'-diaminobenzidine tetrahydrochloride substrate kit (ZSGB-BIO, Beijing, China). Negative controls were performed without primary antibody.

ELISA assay
To investigate the involvement of sex hormone in the plasma concentration of OT, female rats were randomly divided into four groups (n = 6 for each group): gastric instillation with room temperature saline (normal group); gastric instillation with cold saline (cold group); stressed rats which had been ovariectomized before gastric instillation with cold saline (OVX + cold group); ovariectomized rats treated with E_2 (25 μg/kg, s.c.) once daily for 6 d and then gastric instillation with cold saline (OVX + E_2 + cold group). After the animals were anesthetized with sevoflurane, uteri were removed and weighed. The blood from the heart was collected with a syringe into a tube and spun for 10 min at 3000 rpm. Plasma was pipetted out and stored at -80 ℃ for further OT analysis. OT was determined using an Enzyme Immunoassay Kit (RD, Inc., MI, United States) at a dose of 100 μL plasma per sample per well for the assay, according to the manufacturer's instructions. Samples were analyzed in duplicate in a single assay.

Statistical analysis
The peak forces of colonic phasic contraction were measured using an MFlab system (Fudan University, Shanghai, China). In each experiment, the peak forces of contractions were evaluated at 0.5 min before and after drug administration. Mean peak force for the 1-min period before drug administration was taken as the baseline. The value of the force after drug treatment was normalized to the baseline value. The ratio of post-treatment force to baseline force was expressed as the ratio R, so that the baseline for each experiment was equal to 1.

Western blots were evaluated by determining the gray scale value of the blot. The value of ratio is the value of the gray scale division between the experimental group and control group.

Data were presented as means ± SD. Statistical analysis was performed by means of Student's t test for comparisons between two groups or by means of ANOVA analysis for comparisons among groups. A probability level of $P < 0.05$ was considered statistically significant.

RESULTS
Colonic transit test
Figure 1 presents the colonic transit in rats. The time of the glass bead output was 223.08 ± 90.76 s in the cold group and 102.42 ± 20.21 s in the control group. The
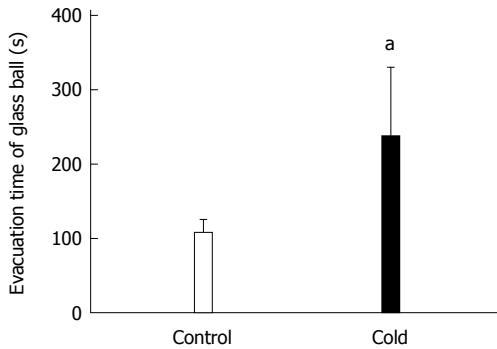
Figure 1 Colonic transit increased in intragastric cold water stressed rats. Control group: the rats treated with intragastric room temperature physiological saline; Cold group: the rats treated with intragastric cold physiological saline. [a]P < 0.05 *vs* cold and control rats (n = 10).
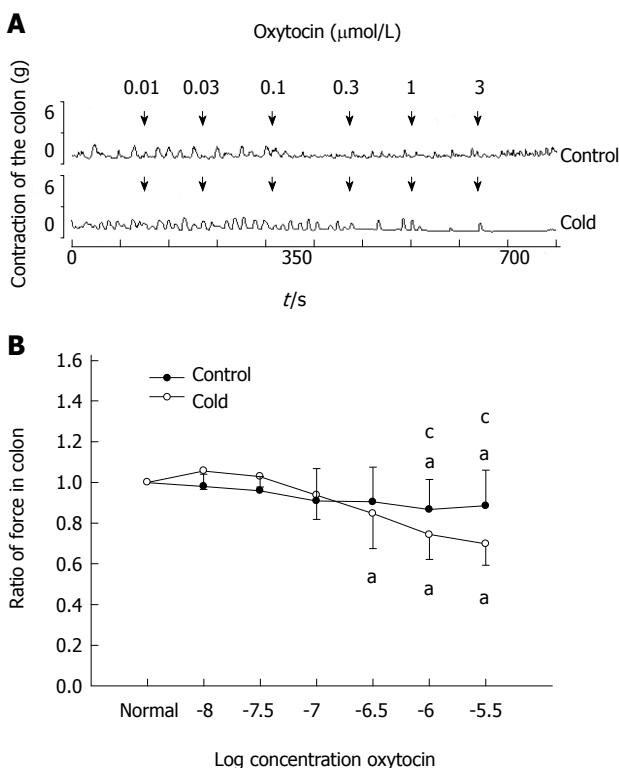
Figure 2 Effects of oxytocin (0.01-0.3 μmol/L) on colon contraction in intragastric cold water stressed rats. A: The dose-dependent contractile response to oxytocin (OT) in colonic smooth muscle strips in cold and control groups. OT was administered at the points marked by the arrows. B: Average response to OT in the colon. [a]P < 0.05 *vs* the data prior to OT administration. [c]P < 0.05 *vs* cold and control groups (n = 6).

colonic transit time in intragastric cold water stressed rats was significantly longer than in control rats (P = 0.029).

Effect of OT on colonic contractility in intragastric cold water stressed rats

As shown in Figure 2, for the control group, low concentrations of OT (0.01-0.3 μmol/L) failed to elicit any effect on the contractions of colonic smooth muscle strips. When the concentration of OT was increased to
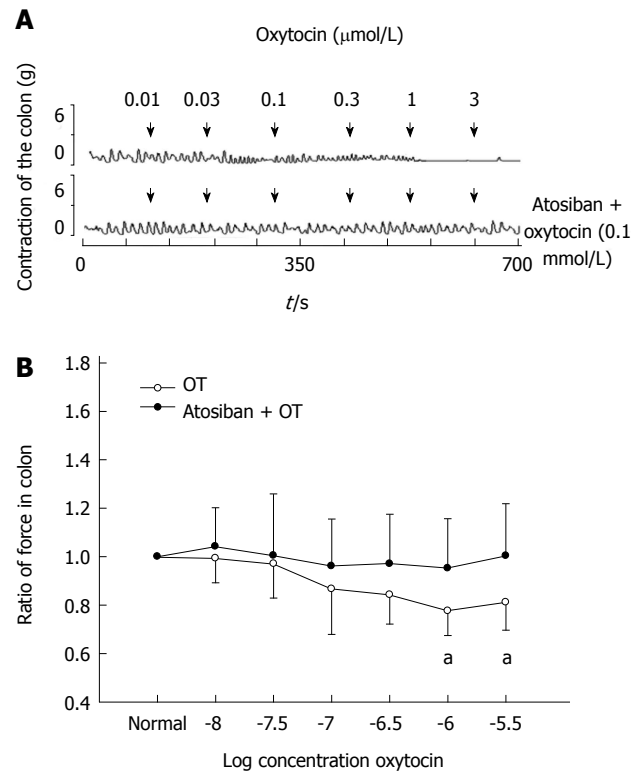
Figure 3 Atosiban (0.1 mmol/L) blocked oxytocin-induced colonic response in intragastric cold water stressed rats. A: Representative traces of the effect of atosiban on oxytocin (OT)-induced colonic response in the cold group. The drug was administered at the points marked by the arrows. B: Results of colonic smooth muscle strips contraction in cold group rats. [a]P < 0.05 *vs* the data of atosiban treatment (n = 8).

1 or 3 μmol/L, the ratio of the contractile force of colonic smooth muscle strips decreased respectively to 0.87 $\pm$ 0.15 or 0.88 $\pm$ 0.16 (P < 0.05 compared with the data prior to OT administration).

In the cold group, OT (0.01-0.1 μmol/L) failed to elicit any effect on the contractions of colonic smooth muscle strips. When the concentration of OT was increased to 0.3, 1 or 3 μmol/L, the ratio of the contractile force of colonic smooth muscle strips decreased respectively to 0.87 $\pm$ 0.15, 0.74 $\pm$ 0.12 or 0.69 $\pm$ 0.08 (P < 0.05 compared with the data prior to OT administration). Moreover, the ratio of the contractile force of colonic smooth muscle strips induced by OT at 1 or 3 μmol/L was lower than that in control group (0.74 $\pm$ 0.12 *vs* 0.87 $\pm$ 0.15, 0.69 $\pm$ 0.08 *vs* 0.88 $\pm$ 0.16, respectively, P < 0.05).

Effect of atosiban on OT-induced response of colon in intragastric cold water stressed rats

OT receptor inhibitor atosiban (0.1 mmol/L) showed no effect on the contraction of colonic strips in stressed rats. Thirty minutes after atosiban treatment, OT (0.01-3 μmol/L) was added. The ratio of the contractile force of colonic smooth muscle strips induced by OT at 1 or 3 μmol/L almost returned to normal (0.72 $\pm$ 0.10 *vs* 0.95 $\pm$ 0.20, 0.78 $\pm$ 0.12 *vs* 1.0 $\pm$ 0.21, respectively, P < 0.05 compared with the data of atosiban treatment, Figure 3).
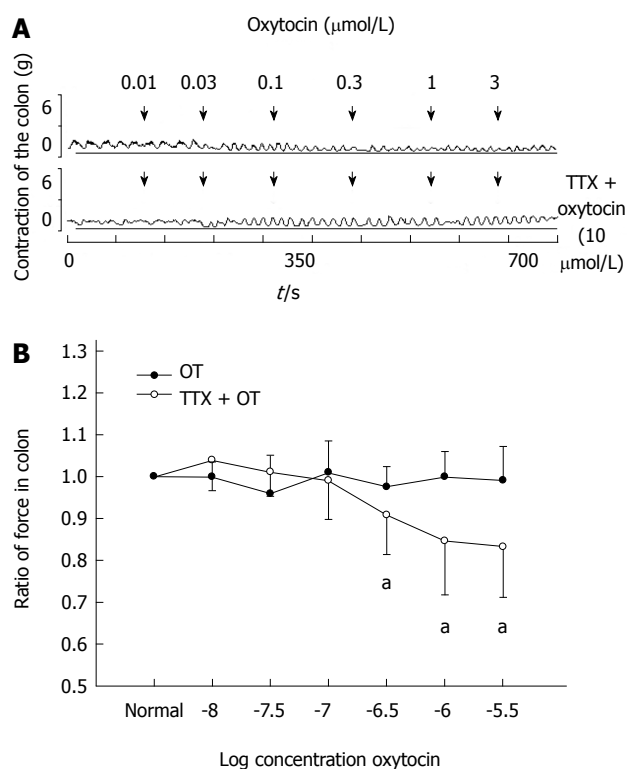
Figure 4 Tetrodotoxin (10 μmol/L) blocked oxytocin-induced colonic response in intragastric cold water stressed rats. A: Representative traces of the effect of tetrodotoxin (TTX) on oxytocin (OT)-induced colonic response in cold group. The drug was administered at the points marked by the arrows. B: Results of colonic smooth muscle strips contraction in cold group rats. [a]$P < 0.05$ *vs* data of TTX treatment ($n = 6$).

Effect of tetrodotoxin on OT-induced response of colon in intragastric cold water stressed rats

Addition of tetrodotoxin (TTX) (10 μmol/L) showed no effect on the contraction of colonic strips in stressed rats. Thirty minutes after TTX treatment, OT (0.01-3 μmol/L) was added. The ratio of the contractile force of colonic smooth muscle strips induced by OT (3 μmol/L) recovered from 0.79 ± 0.13 to 0.99 ± 0.08 ($P < 0.05$ compared with the data of TTX treatment, Figure 4).

OT receptor expression in the colon of intragastric cold water stressed rats

Compared with the control group, the expression level of OT receptor in the colon of stressed rats was significantly increased (1.82 ± 0.17 *vs* 1.00 ± 0.31, $P < 0.05$, Figure 5A, B). The cells with OT receptor immunoreactivity were located in the myenteric plexus of the colon and smooth muscle of the rat uterus (Figure 5C, D).

Plasma concentration of OT in rats

As shown in Figure 6A, the weight of the uteri in OVX rats markedly decreased compared with that of control rats (0.14 ± 0.12 g *vs* 0.46 ± 0.09 g, $P < 0.05$). After the OVX rats were treated with E2, the weight of the uteri recovered to normal (OVX + E2, 0.51 ± 0.10 g, $P < 0.05$ *vs* OVX rats; $P > 0.05$ *vs* control rats).

The plasma concentration of OT was analyzed in
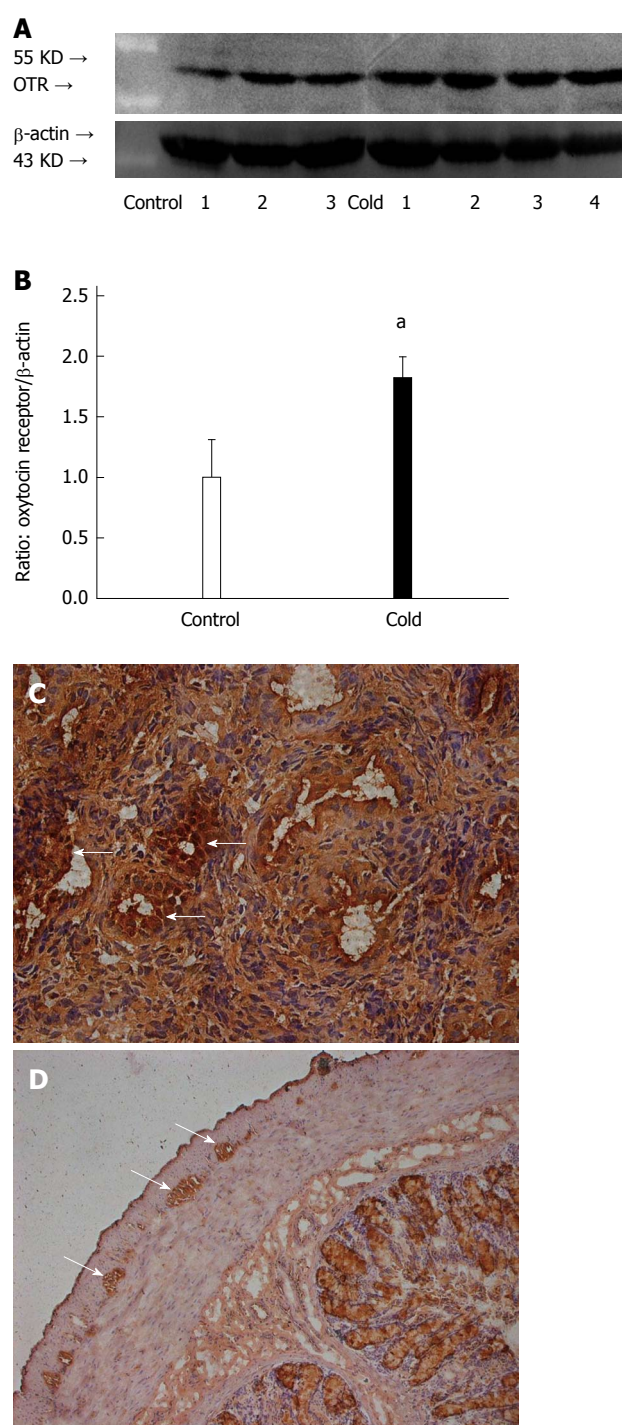
Figure 5 Intragastric cold water stress increased oxytocin receptor expression in colon. A: Representative Western blots. B: Densitometry analysis. C: Positive control of immunoreactivity. Oxytocin (OT) receptor antigen is expressed in the smooth muscle (arrows) of uterus. D: OT receptor immunoreactivity is expressed in myenteric plexus (arrows) of colon. [a]$P < 0.05$ *vs* control group ($n = 6$). OTR: Oxytocin receptors.

these rats. As shown in Figure 6B, the concentration of OT was significantly increased in the cold group compared with the control group (286.99 ± 83.72 pg/mL *vs* 212.42 ± 50.62 pg/mL, $P = 0.036$). After the OVX rats were subjected to gastric instillation with cold saline, the concentration of OT decreased compared with the cold group rats without OVX (100.56 ± 92.71 pg/mL
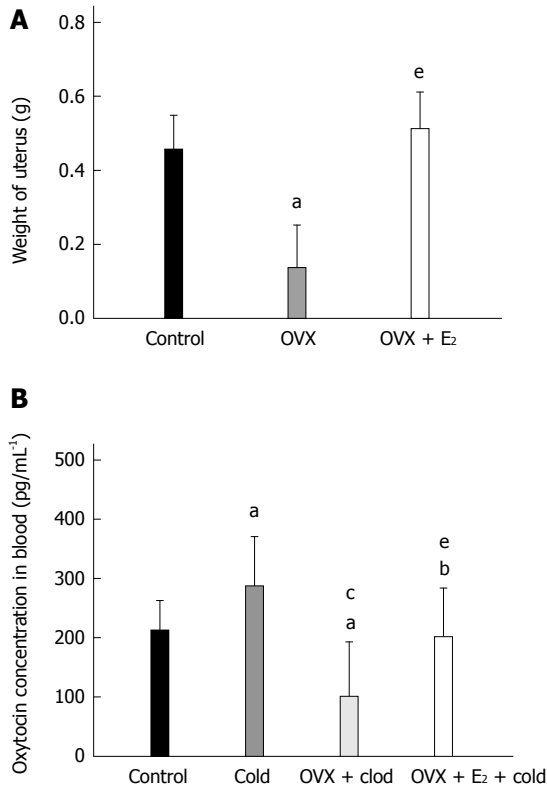
Figure 6 Estradiol regulated the plasma concentration of oxytocin in intragastric cold water stressed rats. A: Weight of uterus. B: The plasma concentration of oxytocin (OT) in the four groups [control, cold, ovariectomy (OVX) + cold and OVX + E$_2$ + cold]. Data are expressed as mean ± SD (n = 6). [a]$P < 0.05$ *vs* control group. [c]$P < 0.05$ *vs* cold group. [e]$P < 0.05$ *vs* OVX + cold group.

Figure 7 Ovariectomy decreased the oxytocin-induced inhibition of colonic contractility in intragastric cold water stressed rats. A: Representative traces of the effect of ovariectomy (OVX) on oxytocin (OT)-induced colonic response in cold group rats. The drug was administered at the points marked by the arrows. OT-induced inhibition of colonic strips decreased after the rats were ovariectomized. B: The contraction of colonic smooth muscle strips in cold group rats. [a]$P < 0.05$ *vs* the data in normal Krebs solution. [c]$P < 0.05$ *vs* cold group and OVX + cold group (n = 6).

vs 286.99 ± 83.72 pg/mL, $P < 0.05$ *vs* the cold group). In OVX + E$_2$ + cold group, the plasma level of OT returned to normal but was still lower than that in the cold group without any treatment (OVX + E$_2$ + cold group, 201.25 ± 80.91 pg/mL; control group, 212.42 ± 50.62 pg/mL; cold group, 286.99 ± 83.72 pg/mL; $P > 0.05$ *vs* control group, $P < 0.05$ *vs* cold group).

Effect of OVX on OT-induced inhibition of colonic contractility in intragastric cold water stressed rats

After the OVX rats were subjected to gastric instillation with cold saline, OT at 1 or 3 μmol/L still decreased the contractile activity of colon (0.84 ± 0.13, 0.83 ± 0.12, respectively, $P < 0.05$ *vs* the data prior to OT administration, Figure 7A, B), but OT-induced inhibition was weaker in the OVX + cold group than that in the cold group (0.84 ± 0.13 *vs* 0.67 ± 0.11, 0.83 ± 0.12 *vs* 0.68 ± 0.12, respectively, $P < 0.05$ *vs* cold group, Figure 7A, B).

DISCUSSION

The present study showed that the colon transit of cold group rats (stomach irritation with cold physiological saline) was longer than that of control group rats (stomach irritation with room temperature physiological saline). Cold irritation enhanced the inhibitory effect of OT on colonic muscle strips in rats. OT has widespread effects on the motility of the gastrointestinal tract. OT has been shown to inhibit gastric emptying and intestinal transit[41], as well as spontaneous contractions of duodenum in rats[30] and colon in rabbits[19]. In contrast, it excites gastrointestinal motility in rabbits[42], and increases gastric spontaneous contraction[29] and colonic contraction in rats[20]. Our previous study showed that exogenous OT decreased the contractions of proximal colonic smooth muscle strips in control mice, while it increased contractions in antenatal maternal hypoxia mice. OT increased the contractions of distal colonic smooth muscle strips in both antenatal maternal hypoxia and control mice[43]. The effects of OT on gastrointestinal motility might depend on the sex of animal, site of gastrointestinal tract, concentration of OT, level of OT receptors or even the stimulation the animal suffered. Cold water intake might increase the sensitivity of colonic smooth muscle to OT and then affect the colonic motility, which is involved in IBS.

Alterations in bidirectional brain-gut interactions are believed to be involved in the pathogenesis of IBS and related functional gastrointestinal disorders. The central nervous system modulates the gastrointestinal tract *via* the sympathetic and parasympathetic branches of the autonomic nervous system and the hypothalamic-pituitary axis[44]. OT mRNA expression in the paraventricular

nucleus was reported to increase following chronic stress in rats[45]. The present study showed that intragastric cold water stress prolonged colon transit. The inhibitory response of colon to OT was enhanced in intragastric cold water stressed rats. OT is essential to a wide range of stress-related disorders[46,47]. OT might act as a brain-gut signal mediator and be involved in IBS by affecting the colonic motility.

We found that OT receptor antagonist atosiban inhibited the effect of OT on colonic motility in intragastric cold water stressed rats. OT acts on the colon *via* OT receptors. The voltage-dependent Na^+ channel antagonist TTX blocked the inhibitory effect of OT in the colon. Our immunoreactivity study showed that OT receptors were located in the myenteric plexus, rather than in colonic smooth muscle cells. Therefore, OT might affect colonic smooth muscle contraction *via* OT receptors in the myenteric plexus. Our study further demonstrated that the expression of OT receptors is up-regulated in intragastric cold water stressed rats. Chronic isolation was reported to regulate plasma OT level and gene expression of OT receptors in the heart of prairie voles[48]. Up-regulation of OT receptors may partially account for the enhanced inhibitory effect of OT on colonic contractility in the cold group.

Following chronic homotypic stress, OT-knockout mice fail to restore accelerated colonic transit compared with wild type mice[49]. Proximal colon distension stimulates OT-containing hypothalamic neurons in rats[50]. We also compared the plasma concentration of OT between the cold group and control group, and found that OT level was significantly higher in the cold group than in the control group. Intragastric cold stress might stimulate the synthesis and release of OT, and then increase the concentration of OT in blood. Increased plasma OT might also be partly responsible for the impaired colon transit in the cold group.

OT and OT receptor mRNA levels are regulated by estrogen and are both reduced due to ovariectomy[51,52]. Estradiol was reported to affect OT neurons and modulate the secretion of OT in response to the increase of osmolality induced by refeeding in rats[53]. For the intragastric cold water stressed rats in our study, the plasma levels of OT decreased in the OVX rats; when the OVX rats were treated with estradiol, the plasma levels of OT were almost the same as the control rats, but were still lower than that of the cold group without any treatment. Estradiol also regulates the secretion of OT in response to intragastric cold stress in rats.

Estrogens facilitate social recognition by regulating OT production and OT receptors[54]. Estradiol modulates the cardiovascular responses induced by hemorrhage *via* enhancement of OT neuron activity[55]. To investigate the involvement of ovarian hormones in OT-induced colonic contraction, the rats were ovariectomized and treated with chronic intragastric cold stress. Our study showed that the response of colonic muscle strips to OT was attenuated in OVX rats. Estradiol facilitates OT-induced colonic contraction in intragastric cold water stressed rats by increasing OT production. OT concentration changes in depressed women, suggesting that OT signaling may provide a mechanism by which to better understand female-biased risk for the development of depressive disorders[56]. OT signaling may also be involved in female-biased risk for IBS.

In conclusion, we found that cold water intake inhibited colonic motility at least partially through oxytocin-oxytocin receptor signaling in the myenteric nervous system pathway, which is estrogen dependent.

COMMENTS

Background
The incidence of irritable bowel syndrome (IBS) is high, predominantly in females, although the cause is largely unknown. Cold meal intake affects gastric myoelectrical and contractile activities. Cold water intake increases plasma 5-HT concentrations in subjects with IBS.

Research frontiers
Oxytocin (OT) is essential to a wide range of stress-related disorders. OT effects are generally related to trait effects related to social or emotional functions, particularly in females. This study suggests that OT might be a potential mechanism involved in IBS.

Innovations and breakthroughs
Cold water intake decreased the colonic transit and OT-induced inhibition of colonic contraction. OT decreased colonic contraction *via* OT receptors in the myenteric plexus. Cold water intake also increased blood concentration of OT. Estradiol regulated blood concentration of OT and OT-induced colonic contraction.

Applications
By understanding how cold water intake affect colonic motility and the involvement of OT in colonic contraction, this study may represent a future strategy for therapeutic intervention in the treatment of patients with IBS.

Terminology
OT is a neuropeptide synthesized by magnocellular neurons in the paraventricular and supraoptic nuclei of the hypothalamus. OT is released from the pituitary and goes into the blood. OT receptors are expressed in colon. OT acts on the OT receptors of the colon and regulates colonic motility. Hypothalamic OT-colonic OT receptor might be an important brain-gut axis to control colonic motility.

Peer review
The authors examined the effect of cold water intake on colonic motility and on OT-induced colonic contraction. The study revealed that cold water intake inhibited colonic motility at least partly through OT-OT receptor signaling in the myenteric plexus pathway, which is estrogen dependent. The experimental design is reasonable and the findings are interesting.

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EVIDENCE-BASED MEDICINE

Electrochemiluminescence immunoassay method underestimates cortisol suppression in ulcerative colitis patients treated with oral prednisone

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Abstract

AIM: To evaluate cortisolemia by using conventional electrochemiluminescence immunoassay (ECLIA) method compared to liquid chromatography-tandem mass spectrometry (LC-MS/MS) method in active ulcerative colitis (UC) patients treated with oral prednisone (PD).

METHODS: Twenty patients (12 males) with acute relapse of UC started oral PD at a dose of 40 mg once a day, tapered of 10 mg every 2 wk. When a stable 2-wk daily dose of 30 mg was reached, blood samples for cortisol levels' measurement were drawn in the morning in fasting conditions to determine circulating cortisol by LC-MS/MS and ECLIA assay.

RESULTS: Median interquartile range cortisolemia with ECLIA and LC-MS/MS method was 54.1 (185.8) nmol/L and 32.1 (124.0) nmol/L, respectively ($P < 0.001$). The within-patient median differences between the two methods was 23.2 (40.6) nmol/L, with higher cortisol levels for the ECLIA method. The estimated geometric mean ratio between methods was 1.85 (95%CI: 2.39-1.43) considering all data or 1.58 (95%CI: 2.30-1.09) considering only data above the limit of quantification (n = 12). The 95%CIs of the geometric mean ratio between methods confirm a statistically significant difference.

CONCLUSION: Blood cortisol levels detected with ECLIA method seems to be higher than the ones measured by LC-MS/MS, indicating a possible overestimation of them in patients treated with PD. Therefore, the cortisol suppression in patients under treatment with oral PD should not be measured using ECLIA method.

Core tip: The determination of morning cortisol levels is used in clinical practice and as specific safety end-point in various clinical trials. This study was designed to compare the efficacy of electrochemiluminescent assays (ECLIA) method and liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for measurement of cortisolemia in active ulcerative colitis (UC) patients treated with oral prednisone (PD). Blood cortisol levels detected with ECLIA method are higher than the ones measured by LC-MS/MS, indicating a possible overestimation of them in UC patients treated with PD. Therefore, the cortisol suppression in patients under treatment with oral PD should not be measured using ECLIA method.

Manguso F, Bennato R, Lombardi G, Viola A, Riccio E, Cipolletta L. Electrochemiluminescence immunoassay method underestimates cortisol suppression in ulcerative colitis patients treated with oral prednisone. *World J Gastroenterol* 2014;

INTRODUCTION

Glucocorticosteroids (GS) are drugs of choice for the management of ulcerative colitis (UC) if symptoms of active colitis do not respond to mesalazine[1]. Patients undergoing repeated administrations of GS may experience a variety of adverse events (AEs), some of which of concerning clinical relevance[2]. Among them, morning cortisol levels below the lower limit of the normal range during treatment may represent the suppression of hypothalamic-pituitary-adrenal (HPA) axis, and is used as specific safety endpoint in the various clinical trials concerning the treatment with GS[3,4].

In the majority of earlier studies, cortisol levels in human biological samples has been determined by immunoassay methods, first radio-immunoassay (RIA), followed by enzyme-linked immunosorbent assay (ELISA), then automated electrochemiluminescent assays (ECLIA) and more recently by liquid chromatographic methods coupled with mass spectrometry detection (LC-MS/MS)[5]. Immunoassays can be affected by cross-reactivity from other steroids and the use of drugs containing GS can positively affect the methods[5,6], depending on the degree of the cross reactivity with the particular assay. Notably, prednisone (PD) and its metabolites are chemically similar to serum cortisol and may strongly interfere with cortisol measurements by immunoassay methods[6]. Analytical methods using chromatographic separation of cortisol from PD and its metabolites, such as LC-MS/MS, may avoid problems of interference[7].

Concentrations of cortisol may be overestimated by using conventional ECLIA method in samples from UC patients who are treated with PD. Therefore, the aim of the study was to compare the blood cortisol levels measured using ECLIA and a selective LC-MS/MS method in samples collected from patients with UC treated with oral PD.

MATERIALS AND METHODS

Patients and procedures

In a prospective study (ELICA Study: ACTRN12610000425099) 20 patients of both sexes, aged between 18 and 70 years, with acute relapse of UC uncontrolled with mesalazine alone were included. All patients had an extension of the disease above the rectum and indication for systemic CS treatment. The exclusion criteria were proctitis, ongoing local (enema) or systemic treatment with CS within 3 mo, low compliance to medical treatment and presence of other diseases or treatments that are known to interfere with the evaluation of blood cortisol concentrations as a measure of HPA axis.

The study included a screening visit for study presentation and signature of informed consent, collection of demographic data, medical history and concomitant treatment, together with physical examination and vital signs, inclusion/exclusion criteria check and assignment of a patient identification number. The extension of UC was reported according to Montreal classification[8].

Following a minimum of 3-d run-in period, inclusion/exclusion criteria were confirmed and oral PD treatment (Deltacortene®, 5 and 25 mg tablets, Bruno Farmaceutici, Italy) was started at a dose of 40 mg once a day with a decalage of 10 mg every 2 wk. An appointment for blood sampling visit was given at the end of the first 4 wk of treatment, when a stable 2-wk daily dose of 30 mg was reached.

Blood sampling visit included the recording of steroid-related AEs and blood drawn of about 10-12 mL (about 8 mL in serum vials and about 2-4 mL in EDTA-coated vials) in fasting conditions at a 24-h distance from last PD dose (between 8 AM and 9 AM).

Cortisol sampling

For serum preparation, blood samples were collected by direct venipuncture or via an indwelling cannula in the forearm into the serum vacutainer coagulating for 30 min, protected from light, and centrifuged for 10 min at 1300 g/room temperature. Afterwards, serum was transferred each 1.5 mL of the clear supernatant into two sample tubes using a disposable pipette. For plasma preparation, blood samples were collected into vacuum tubes containing EDTA as anticoagulant, and centrifuged within 15 min after collection. The plasma was separated in a centrifuge at 1500 *g* for 10 min. After each centrifugation, the supernatant (plasma) was dispensed in labeled polypropylene tubes, using Pasteur pipettes. The plasma was dispensed equally between the two polypropylene tubes (minimum of 1 mL of plasma per tube). Once collected, the plasma samples were immediately stored vertically below -20 ℃.

Serum (from untreated blood) was used to determine circulating cortisol by ECLIA assay, and plasma (from EDTA-treated blood) was used to determine circulating cortisol by LC-MS/MS assay. One aliquot of serum and one aliquot of plasma were stored as backup for possible re-analysis. Specimens were stored at -20 ℃ at the investigational site until shipment to two different laboratories for cortisol assay. The analytical work was conducted in two specialized laboratories. Plasma specimens were shipped to the laboratory of the Mass Spectrometry Division at SGS Life Science Services, Wavre, Belgium, while serum specimens to the laboratories at INTERLAB GmbH, Munich, Germany. Biological samples were sent to the laboratories conducting the assays in insulated containers filled with a sufficient amount of coolant material. In all cases, cortisol concentrations were reported in units of nmol/L, and values < 171.1 nmol/L were considered below normal.

Serum cortisol was measured using the Cortisol assay (Roche Diagnostics) following the manufacturer's instruc-

tion[9]. Serum cortisol concentrations were determined using an electrochemiluminescence immunoassay on a Roche Cobas analyzer. The lower and upper limits of measurements were 0.5 and 1750 nmol/L, respectively.

Plasma cortisol was measured by SGS Life Science Services with a validated LC-MS/MS method. The methodology and its validation are described in SGS Life Science Services' validation report no. B090806 entitled 'Validation of a LC-MS/MS method for the determination of cortisol in EDTA human plasma'. Cortisol levels were detected using an Api 4000 mass spectrometer (AB Sciex). Computer application programs used to acquire and derive data for this study included the validated Thermoelectron Corporation Watson version 6.4, and MDS Sciex Analyst® 1.4.1 version. The acceptance criteria for the carry-over, the calibration samples, the QCs as well as the criteria for re-assay, correspond to the Standard Operating Procedures in use in the laboratory conducting the assays. Seven samples, arranged in increasing sequence, were used for calibration, with the first concentration level corresponding to the lower limit of quantification (LLOQ). The lower and upper limits of measurements were 27.6 and 829 nmol/L, respectively. The precision of the method as characterized by the coefficient of variation (CV%) for plasma quality control samples ranged between 4.28% and 10.8%. The method proved to be selective against prednisone and its metabolites prednisolone and 16-α-hydroxyprednisolone.

Primary evaluation parameter was the difference between cortisol concentrations assayed with the two analytical methods.

Ethical considerations
The study was approved by the Local Ethics Committee of A. Cardarelli Hospital (Napoli, Italy) and all patients gave written informed consent prior to any study related procedures.

Statistical analysis
For descriptive analyses, continuous variables are presented as mean ± SD or median interquartile range (IQR) according to the Gaussian distribution. Wilcoxon signed ranks test was used to compare the two related cortisol samples, and differences between the two analytical methods were graphically showed according to Bland and Altman suggestions[10]. Values below LLOQ obtained with the LC-MS/MS method were numerically set at ½ LLOQ. Data were log$_e$ transformed and showed as simple scatterplot with the line of the equality represented. Moreover, in all patients and in those with data above the limit of quantification, the antilog of the difference between the two methods (log$_e$ ECLIA - log$_e$ LC-MS/MS) was plotted against the antilog of the average [(log$_e$ ECLIA + log$_e$ LC-MS/MS)/2] showing the geometric mean ratio with upper and lower 95%CI. Statistical analysis was performed using SAS 9.1.3 software (SAS Institute®, Cary, NC, United States).

Table 1 Patients' characteristics

Characteristics	Data
Age, yr	37.9 ± 16.2
Range	20-70
Male/females, *n*	12/8
Disease localization, *n* (%)	
Left colitis	11 (55)
Pancolitis	9 (45)
Years from diagnosis, median (IQR)	4 (9)
Height, cm	166.7 ± 7.7
Weight, kg	68.8 ± 15.2
BMI	24.7 ± 5.3
SBP, mmHg	113.9 ± 7.9
DBP, mmHg	71.3 ± 5.6
HR, bpm	76.5 ± 9.2

Data are mean ± SD except when indicated. IQR: Interquartile range; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; HR: Heart rate; bpm: Beats per minute.

RESULTS
The main demographic characteristics of the recruited subjects are reported in Table 1. At the end of the study period two patient (10%) presented with steroid related AEs not including cortisol levels below normal values. Higher values of cortisol levels were found for ECLIA than LC-MS/MS method ($P < 0.001$). Median (IQR) blood cortisol levels with ECLIA were 54.1 (185.8) nmol/L, while with LC-MS/MS assay 32.1 (124.0) nmol/L. The within-patient median differences between the two methods was 23.2 (40.6) nmol/L and the ECLIA/LC-MS/MS ratio 1.7 (1.3), reflecting higher cortisol levels measured with the ECLIA method. Mean percentage difference between the results obtained with the two methods was of 39%.

As showed in Figure 1, all values obtained with the ECLIA method were below the line of equality suggesting a systematic over-estimation with this method. The estimated geometric mean ratio between methods, based on differences of log$_e$ transformed data, was 1.85 (95%CI: 2.39-1.43) considering all data (Figure 2) or 1.58 (95%CI: 2.30-1.09) considering only data above the limit of quantification ($n = 12$) (Figure 3). The 95%CIs of the geometric mean ratio between methods confirm a statistically significant difference.

DISCUSSION
The risk of underestimation of the suppression of endogenous cortisol production in patients treated with prednisone, when conventional RIA methods are used to detect cortisol levels, is reported in asthmatic patients and is related to cross reactivity of prednisone and its metabolites with cortisol[6]. No data are reported in patients affected by UC.

We tested the degree of interferences of the ECLIA Cortisol assay method when analyzing samples collected from patients treated with prednisone, finding that corti-
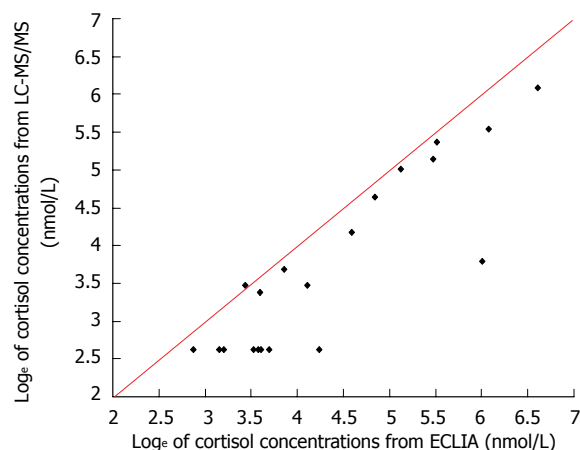
Figure 1 Cortisol concentrations plot with electrochemiluminescence immunoassay *vs* liquid chromatography-tandem mass spectrometry log$_e$-transformed data. LC-MS/MS: Liquid chromatographic methods coupled with mass spectrometry detection; ECLIA: Electrochemiluminescence immunoassay.

Figure 2 Difference between electrochemiluminescence immunoassay and liquid chromatography-tandem mass spectrometry methods using log$_e$-transformed data. LC-MS/MS: Liquid chromatographic methods coupled with mass spectrometry detection; ECLIA: Electrochemiluminescence immunoassay.

Figure 3 Difference between electrochemiluminescence immunoassay and liquid chromatography-tandem mass spectrometry methods using log$_e$-transformed data in the 12 patients with cortisol levels below the limit of quantification. LC-MS/MS: Liquid chromatographic methods coupled with mass spectrometry detection; ECLIA: Electrochemiluminescence immunoassay.

preferred.

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COMMENTS

Background
The determination of morning cortisol levels during treatment with glucocorticosteroids (GS) is used in clinical practice and as specific safety endpoint in the various clinical trials concerning the treatment with GS. In samples from patients who have been treated with prednisone (PD), concentrations of cortisol may be overestimated by using conventional electrochemiluminescence immunoassay (ECLIA) method.

Research frontiers
Data about the degree of possible differences between ECLIA and liquid chromatographic methods coupled with mass spectrometry detection (LC-MS/MS) methods in the determination of cortisol levels in ulcerative colitis (UC) patients in treatment with PD are lacking.

Innovations and breakthroughs
This is the first study comparing blood cortisol levels measured using ECLIA and a selective LC-MS/MS method in samples collected from patients with UC treated with oral PD.

Applications
Data obtained with the ECLIA method are higher than the ones measured by LC-MS/MS, indicating overestimation of cortisol levels in patients treated with PD. The authors conclude that the cortisol suppression in the presence of PD should not be assessed by ECLIA method.

Terminology
Cortisol levels in human biological samples has been determined by immunoassay methods, first radio-immunoassay, followed by enzyme-linked immunosorbent assay, then automated ECLIA and more recently by LC-MS/MS.

Peer review
For some scientific researches, which focus on the depression effect of glucocorticosteroid on hypothalamic-pituitary-adrenal axis in UC patients, this study may provide useful information.

sol levels measured using ECLIA method were 39% higher than the ones measured by the selective LC-MS/MS method. These data indicate an overestimation of cortisol levels and an underestimation of the HPA axis suppression using ECLIA method in patients with UC treated with PD. When assessing cortisol suppression by systemic corticosteroids, we believe that researchers and clinicians might not be aware of this problem or the extent of it. In large-scale clinical trials on UC patients treated with PD where HPA axis suppression is a safety endpoint, the results may be biased by the determination of serum cortisol levels with RIA or ECLIA methods. Selective analytical method against prednisone and its metabolites prednisolone and 16-α-hydroxyprednisolone LC-MS/MS should be used in this clinical setting to avoid the interference of PD in the determination of cortisol levels.

In conclusion, the use of ECLIA method to assess the effects of PD on cortisol suppression could be misleading and the use of LC-MS/MS evaluation should be

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EVIDENCE-BASED MEDICINE

Criteria-specific long-term survival prediction model for hepatocellular carcinoma patients after liver transplantation

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Abstract

AIM: To establish a model to predict long-term survival of hepatocellular carcinoma (HCC) patients after liver transplantation (MHCAT).

METHODS: Two hundred and twenty-three patients with HCC were followed for at least six years to identify independent risk factors for long-term survival after liver transplantation (LT). The criteria for HCC liver transplantation included the Milan, University of California San Francisco, Hangzhou and Shanghai Fudan criteria. The Cox regression model was used to build MHCAT specifying these criteria. A survival analysis was carried out for patients with high or low risk.

RESULTS: The one-, three- and five-year cumulative survival of HCC patients after LT was 78.9%, 53.2% and 46.4%, respectively. Of the HCC patients, the proportion meeting the Hangzhou and Fudan criteria was significantly higher than the proportion meeting the Milan criteria (64.6% *vs* 39.5%, 52.0% *vs* 39.5%, $P < 0.05$). Moreover, the proportion meeting the Hangzhou criteria was also significantly higher than the proportion meeting other criteria ($P < 0.01$). Pre-operative alfa-fetoprotein level, intraoperative blood loss and retransplantation were common significant predictors of long-term survival in HCC patients with reference to the Milan, University of California San Francisco and Fudan criteria, whereas in MHCAT based on the Hangzhou criteria, total bilirubin, intraoperative blood loss and retransplantation were independent predictors. The *c*-statistic for MHCAT was 0.773-0.824, with no statistical difference among these four criteria. According to the MHCAT scoring system, patients with low risk showed a higher five-year survival than those with high risk ($P < 0.001$).

CONCLUSION: MHCAT can effectively predict long-term survival for HCC patients, but needs to be verified by multi-center retrospective or randomized controlled trials.

Key words: Criteria; Hepatocellular carcinoma; Liver transplantation; MHCAT; Survival model

Core tip: This study was conducted to establish a model to predict long-term survival of hepatocellular carcinoma (HCC) patients after liver transplantation (MHCAT) with reference to different criteria and peri-transplant risk factors. We found that MHCAT can effectively predict long-term survival for HCC patients, but needs to be verified by multi-center retrospective or randomized controlled trials.

INTRODUCTION

Liver transplantation (LT) is now a widely accepted treatment for patients with hepatocellular carcinoma (HCC), curing both the underlying disease and the cancer. HCC is currently an indication for LT, which accounts for 16% and 20.9% of all cases in Europe and the United States, respectively[1,2]. In China, the rate is as high as 40%[3]. The progression of HCC and its prognosis after LT are quite different from benign end-stage liver diseases and the success of LT for HCC depends largely on the tumor load, such as size, number of lesions and biological activities. Therefore, the Milan criteria (a solitary HCC nodule 5.0 cm or less in diameter, or no more than three tumor nodules with the largest lesion 3.0 cm or less in diameter, without tumor invasion of blood vessels or lymph nodes) were introduced to achieve a compatible post-transplant survival for HCC and other indications for LT[4,5]. This has also led to controversy regarding the extension of criteria boundaries, such as the University of California San Francisco (UCSF) criteria (similar to the Milan criteria, extending the diameter to 6.5 cm for a solitary nodule and 4.5 cm for the largest, and 8.0 cm as the total when multiple nodules are present)[6,7], Shanghai Fudan criteria (similar to the UCSF criteria, extending the diameter to 9.0 cm for a solitary nodule and 5.0 cm for the largest, and 9.0 cm as the total when multiple nodules are present) and Hangzhou criteria (a total tumor diameter 8 cm or less or total tumor diameter more than 8 cm, with Edmondson grade I or II and pre-operative alfa-fetoprotein (AFP) level 400 ng/mL or less, simultaneously)[8,9]. In view of the global shortage of organ donation, it is critical to achieve a balance between not only a waiting list and post-transplant survival, but also benefit in HCC patients and other recipients. This study was conducted to build a model to predict long-term survival of HCC patients after LT with reference to different criteria and peritransplant risk factors.

MATERIALS AND METHODS

Subjects and data collection

We followed 223 HCC patients who received deceased donor LT from January 2001 to December 2006 at Changzheng Hospital (Shanghai, China), including four cases of retransplantation due to HCC recurrence. Patients with cholangiocellular carcinoma, mixed liver cancer and malignancies discovered incidentally during transplant were excluded from the study. The follow-up of all 223 cases started on the day of LT until death,

Table 1 Characteristics of hepatocellular carcinoma patients and univariate assessment of long-term survival after liver transplantation *n* (%)

Variables	Value	*P* value
Age (yr)	47.99 ± 9.09	< 0.001
Gender		0.048
Male	204 (91.5)	
Female	19 (8.5)	
Blood type		0.951
A	66 (29.6)	
B	62 (27.8)	
O	75 (33.6)	
AB	20 (9.0)	
Identical	195 (87.4)	0.492
History		
Cardiovascular disease	17 (7.6)	0.358
Respiratory disease	3 (1.3)	0.296
Diabetes mellitus	17 (7.6)	0.736
Hepatitis B virus infection	223 (100.0)	0.252
Ascites	144 (64.6)	0.426
Variceal bleeding	25 (11.2)	0.023
Encephalopathy	4 (1.8)	0.799
Treatment related to hepatocellular carcinoma		
Hepatectomy	21 (9.4)	0.693
Transplantation	4 (1.8)	< 0.001
TACE	44 (19.7)	0.756
Criteria		
Milan		< 0.001
Met	88 (39.5)	
Exceeded	135 (60.5)	
UCSF		< 0.001
Met	97 (43.5)	
Exceeded	126 (56.5)	
Shanghai Fudan		< 0.001
Met	116 (52.0)	
Exceeded	107 (48.0)	
Hangzhou		< 0.001
Met	144 (64.6)	
Exceeded	79 (35.4)	
MELD score	14.12 ± 7.02	0.086
SCr (μmol/L)	71.41 ± 69.10	0.173
TB (μmol/L)	89.45 ± 174.19	< 0.001
INR	1.42 ± 0.45	0.010
AFP (ng/mL)	5694.77 ± 12584.00	< 0.001
Cold ischemia time (h)	9.21 ± 2.07	0.129
Intraoperative blood loss (IU)	9.24 ± 8.50	0.002
Operation duration (h)	8.29 ± 1.58	0.200
Liver transplantation technique		0.001
Classic	212 (95.1)	
Piggyback	11 (4.9)	
Biliary reconstruction		< 0.001
Duct-to-duct	221 (99.1)	
Roux-en-Y	2 (0.9)	
Edmondson grading		0.790
I	8 (3.6)	
II	200 (89.7)	
III	13 (5.8)	
IV	2 (0.9)	

MELD: Model for end-stage liver disease; SCr: Serum creatinine; TB: Total bilirubin; INR: International normalized ratio; AFP: Alfa-fetoprotein; UCSF: University of California San Francisco; TACE: Transcatheter arterial chemoembolization.

retransplantation or the end of the study (December 31, 2012).

Follow-up ended at the second transplantation in some recipients. This was to avoid the halo effect of

retransplantation on modeling survival after the first LT. Pre- and intra-operative potential risk factors and criteria for HCC are listed in Table 1. All data included in the final analysis were extracted from the records of our center in the China Liver Transplant Registry. Ethical approval for the use of human subjects was obtained from the Research Ethics Committee of Changzheng Hospital, consistent with the ethical guidelines of the 1975 Declaration of Helsinki. Informed consent was obtained from each patient.

Immunosuppressive protocol and follow-up

The post-LT immunosuppressive protocols were tacrolimus or cyclosporine, mycophenolate mofetil and steroids. Steroids were usually tapered and withdrawn within the first month after LT. Follow-up was routinely conducted in the outpatient clinics. Patients were followed up every 2 mo during the first postoperative year and at least every 3 to 4 mo thereafter. All patients were monitored prospectively by serum AFP, abdominal ultrasonography, and chest X-ray every 1 to 6 mo, according to the postoperative time. For patients with test results suggestive of recurrence, computed tomography (CT) and/or magnetic resonance imaging (MRI) were used to verify whether intrahepatic recurrence and/or distal metastasis had occurred. A diagnosis of recurrence was based on typical imaging appearance on CT and/or MRI scan and an elevated AFP level.

Statistical analysis

Data were analyzed using statistical software (PASW Statistics@ 18; Chicago, IL, United States). Continuous variables were reported as mean ± SD or as median (range, minimum to maximum) if the variable was not normally distributed. Categorical variables were given as frequencies (%). The primary outcome was death or retransplantation of patients. The patients remained at risk as long as they were free from recurrence and alive during the follow-up. Kaplan-Meier analysis with the log-rank test and Cox proportional hazards model were used for time-to-event analysis. Covariate selection was a non-automated form of backward elimination. An adjusted hazard ratio, together with 95%CI, was used as the risk measurement for mortality. The receiver operating characteristic (ROC) curve was used to determine the efficacy of the survival models, and the area under the ROC curve (c-statistic) was compared using Kruskal and Wallis analysis. P values and 95%CIs were estimated in a two-tailed manner. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Characteristics of the subjects

All LTs were ABO type compatible. Among the 223 cases of HCC undergoing LTs, 135 were beyond the Milan criteria. During the period between January 2001 and December 2006, we performed 502 LTs in total. As 44.4%

of the 502 patients had HCC, HCC was considered a major indication for LT in our center. These patients were followed for 46.31 mo on average (range, 0.03-118.25 mo). The one-, three- and five-year cumulative survival of HCC patients after LT was 78.9%, 53.2% and 46.4%, respectively. The one-, three- and five-year HCC recurrence-free survival after LT was 77.4%, 52.6% and 45.8%, respectively. Of the HCC patients in our study, the proportion meeting the Hangzhou and Fudan criteria was significantly higher than the proportion meeting the Milan criteria (64.6% *vs* 39.5%; 52.0% *vs* 39.5%, $P < 0.05$). Moreover, the proportion meeting the Hangzhou criteria was also significantly higher than the proportion meeting other criteria ($P < 0.01$). Demographic and clinical characteristics are summarized in Table 1.

Univariate analysis

A univariate analysis was conducted using the Cox proportional hazards model. This analysis showed that the predictors significantly affecting long-term survival included age, gender, history of variceal bleeding, retransplantation, total bilirubin (TB), international normalized ratio, AFP, intraoperative blood loss, LT technique (classic or piggyback), biliary reconstruction pattern and four HCC LT criteria; the P values of which were all < 0.05.

Constructing MHCAT based on different criteria

The characteristics presented in the plurality and the lowest value was used as the reference group for qualitative and quantitative data, respectively. In particular, HCC meeting the indication criteria was used as a reference.

All risk factors that were significant in the univariate analysis were entered into the Cox proportional hazard model and the final MHCAT was built using a backward selection procedure. Intraoperative blood loss, retransplantation and AFP level were common significant predictors for survival of five years in HCC patients after LT with reference to Milan, UCSF, and Shanghai Fudan criteria, whereas in MHCAT based on the Hangzhou criteria, TB, intraoperative blood loss and retransplantation were independent predictors (Table 2). ROC curves were generated for the MHCAT scoring system (Figure 1). The area under the ROC curves for MHCAT based on the Milan, UCSF, Fudan and Hangzhou criteria was 0.818 (95%CI: 0.763-0.872), 0.824 (95%CI: 0.771-0.878), 0.811 (95%CI: 0.755-0.867) and 0.773 (95%CI: 0.711-0.835), respectively. These high areas under the ROC values, of which there was no significant difference using the Kruskal and Wallis test ($P > 0.05$), showed that MHCAT had a good performance in predicting five-year post-transplant survival of HCC patients. The MHCAT cut-off value with reference to the Milan, UCSF, Fudan and Hangzhou criteria was 1.749 (sensitivity 0.821; specificity 0.320), 1.714 (sensitivity 0.813; specificity 0.300), 1.152 (sensitivity 0.754; specificity 0.250) and 1.295 (sensitivity 0.642; specificity 0.130), respectively. Patients were divided into high-risk or low-risk groups according to these four cut-off values. Of 223 HCC cases, the number of cases in

Table 2 Long-term survival model for hepatocellular carcinoma patients after liver transplantation

Variables	Regression coefficient	Regression coefficient SE	P value	Hazard ratio	95%CI
Milan[1]					
AFP (logevalue)	0.093	0.031	0.003	1.097	1.032-1.167
Intraoperative blood loss	0.038	0.009	< 0.001	1.039	1.021-1.057
Retransplantation	1.429	0.522	0.006	4.173	1.501-11.604
Criteria exceeded	1.504	0.253	< 0.001	4.500	2.741-7.386
University of California San Francisco[2]					
AFP (logevalue)	0.090	0.031	0.003	1.094	1.030-1.161
Intraoperative blood loss	0.038	0.009	< 0.001	1.039	1.022-1.057
Retransplantation	1.373	0.522	0.009	3.947	1.419-10.976
Criteria exceeded	1.555	0.242	< 0.001	4.737	2.950-7.607
Shanghai Fudan[3]					
AFP (logevalue)	0.091	0.031	0.003	1.096	1.031-1.165
Intraoperative blood loss	0.034	0.008	< 0.001	1.035	1.018-1.052
Retransplantation	1.296	0.523	0.013	3.654	1.311-10.182
Criteria exceeded	1.361	0.213	< 0.001	3.899	2.566-5.924
Hangzhou[4]					
TB (logevalue)	0.186	0.094	0.049	1.204	1.001-1.449
Intraoperative blood loss	0.020	0.010	0.046	1.020	1.000-1.040
Retransplantation	1.312	0.520	0.012	3.715	1.340-10.295
Criteria exceeded	1.520	0.190	< 0.001	4.570	3.149-6.633

[1]MHCAT (Milan) = 0.093 × LnAFP + 0.038 × IBL + 1.429 × Re (Re = 0 without retransplantation; Re = 1 with retransplantation) + 1.504 × Cri (Cri = 0 within criteria; Cri = 1 exceeding criteria); [2]MHCAT (University of California San Francisco) = 0.090 × LnAFP + 0.038*IBL + 1.373 × Re (Re = 0 without retransplantation; Re = 1 with retransplantation) + 1.555 × Cri (Cri = 0 within criteria; Cri = 1 exceeding criteria); [3]MHCAT (Fudan) = 0.091 × LnAFP +0.034 × IBL + 1.296 × Re (Re = 0 without retransplantation; Re = 1 with retransplantation) + 1.361 × Cri (Cri = 0 within criteria; Cri = 1 exceeding criteria); [4]MHCAT (Hangzhou) = 0.186 × LnTB + 0.020 × IBL + 1.312 × Re (Re = 0 without retransplantation; Re = 1 with retransplantation) + 1.520 × Cri (Cri = 0 within criteria; Cri = 1 exceeding criteria). TB: Total bilirubin; AFP: Alfa-fetoprotein.
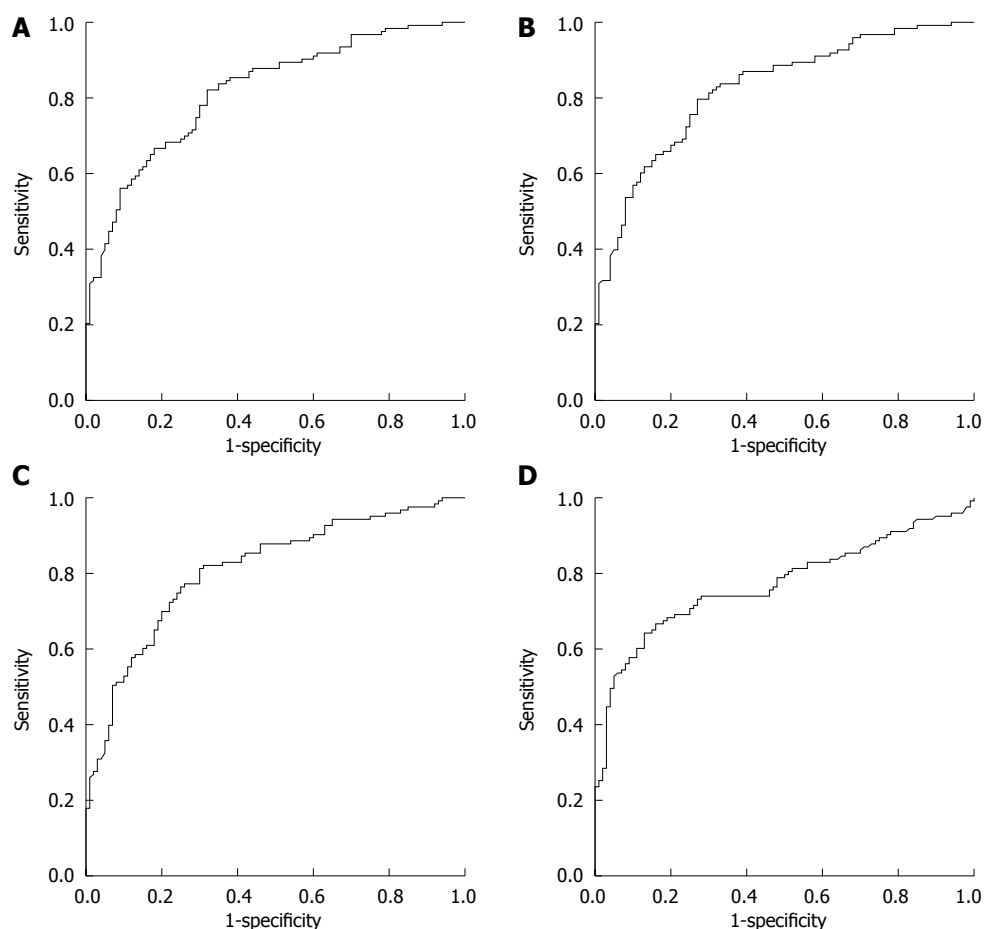
Figure 1 Receiver operating curve for model to predict long-term survival of hepatocellular carcinoma patients after liver transplantation scoring system. A: Milan criteria; B: University of California San Francisco criteria; C: Shanghai Fudan criteria; D: Hangzhou criteria.
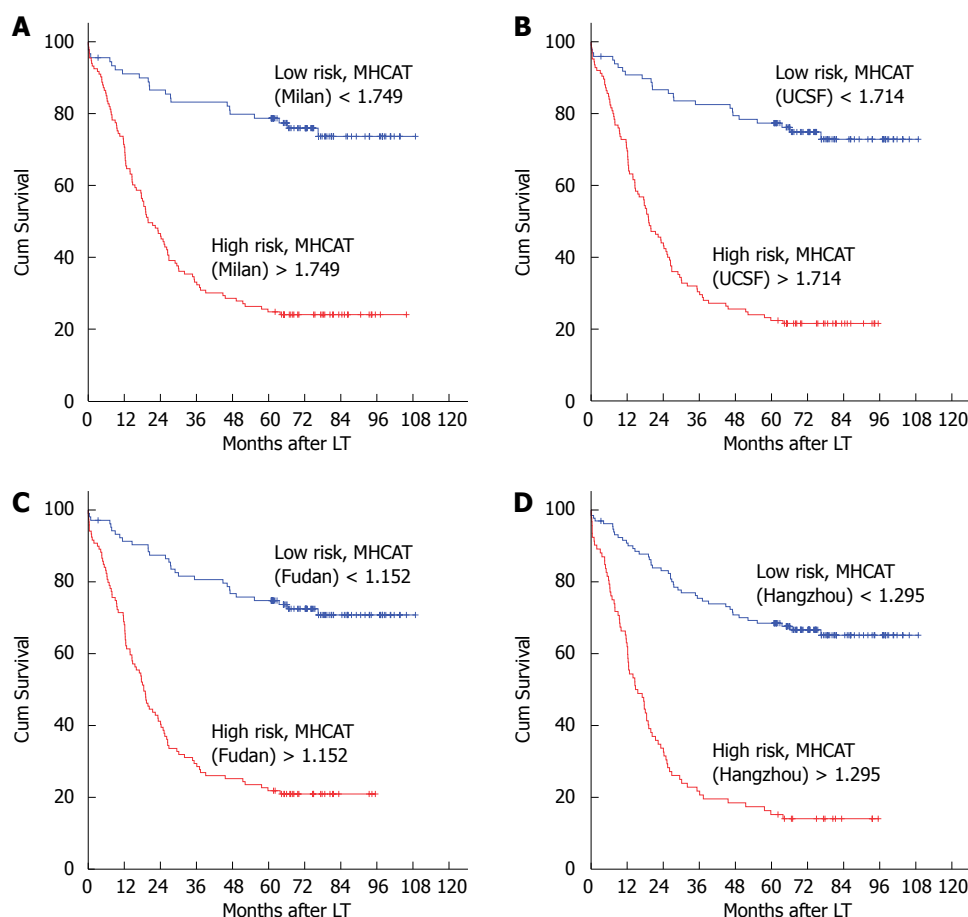
Figure 2 Survival analysis based on cut-off values for the model to predict long-term survival of hepatocellular carcinoma patients after liver transplantation scoring system (P < 0.01 for all). A: Milan criteria; B: University of California San Francisco criteria; C: Shanghai Fudan criteria; D: Hangzhou criteria.

the low-risk group with reference to MHCAT based on the Milan, UCSF, Fudan and Hangzhou criteria was 91, 93, 104 and 121, respectively. There were more HCC patients in the low-risk group under the Hangzhou criteria that under the Milan and UCSF criteria (P < 0.05). Irrespective of the criteria adopted, Kaplan-Meier analysis showed a significantly higher long-term survival in low-risk patients compared with high-risk patients (Figure 2, P < 0.001).

DISCUSSION

Over the past decade, the gap in LT expertise between developing and developed countries has significantly narrowed. With a total number of LTs of more than 26000 cases, China is now second only to the United States. However, the five-year survival after LT in China is significantly lower than that in the United States and Europe (60.5% *vs* 73.7% and 60.5% *vs* 73.0%, respectively)[1-3]. However, the difference in survival of patients with benign end-stage liver diseases is smaller: 73.2% in China, 74.1% in America and 73.2% in Europe. A low curative effect is mainly responsible for the low survival rate of HCC patients in China as compared with those in the United States and Europe (49.7% *vs* 67.5% and 49.7% *vs* 64.0%, respectively). In China, 65.7% of HCC patients

exceeded the Milan criteria before transplantation, which inevitably adversely affected their long-term survival. A multicenter evaluation showed that allocation strategies and different regions could also affect long-term survival after LT[10]. The MHCAT was built with reference to the four most representative HCC LT criteria, using accurate HCC patient data from a single center in China with a follow-up of at least six years. This model may help clinicians determine which candidates with HCC should receive LT.

LT produces excellent results in HCC patients within the Milan criteria. These recipients showed a five-year survival of up to 70% after LT and HCC recurrence was lower than 10%[4]. In recent years, some groups have argued that the Milan criteria are too restrictive and exclude some HCC patients from LT despite the possibility of benefit. Apart from the four criteria included in our study, there are many other criteria for HCC LT, such as the Up-to-Seven criteria, Pittsburgh criteria, and Navarra criteria[11]. However, we considered that the Milan, UCSF, Fudan and Hangzhou criteria best represented the HCC LT criteria. The Milan criteria are now the most widely accepted criteria, recommended by the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases. The Milan criteria are also the basis of other expanded criteria and

the standard reference in studies related to HCC LT criteria. The UCSF criteria are the first expanded criteria and the most studied, involving the largest number of HCC patients. In China, 65.7% of HCC patients exceeded the Milan criteria before transplantation, which is quite different from the United States and European countries. Therefore, we believe that it was reasonable to include criteria based on HCC patients in China in the MHCAT study. The Fudan criteria were chosen because they were proposed according to multicenter data. Moreover, the Hangzhou criteria first included the AFP level and Edmondson staging, which were quite different from other criteria based on tumor morphology.

Several studies have shown that prognostic factors for HCC LT include not only tumor load, such as number of lesions, size and vascular invasion, but also characteristics of the biological activities in the tumor, such as the level and dynamic change in AFP, tumor progression after transcatheter arterial chemoembolization, as well as tumor recurrence after hepatectomy[6,12-14]. Our study on MHCAT also found that the pre-transplant AFP level was an independent prognostic factor, and other factors, such as intraoperative blood loss, retransplantation and TB, may play critical roles in the long-term survival of HCC patients. Therefore, the allocation system based on the model for end-stage liver disease (MELD) has its defects when giving additional priority to HCC patients, as it cannot take all these factors into account. Consequently, it might give rise to controversy and ethical concerns when considering the rights of other recipients.

The goal of LT, regardless of the underlying disease, is to provide liver recipients with the maximum benefit possible from limited resources of donated organs in a fair, ethical, and cost-effective manner. Rules for the distribution of donor organs are closely supervised by all stakeholders involved in LT. Thus, survival models, especially for HCC patients, which may offset the disadvantages of the MELD scoring system, have been an area of research focus. A national conference on liver allocation to patients with HCC in the United States achieved a general consensus for the development of a calculated continuous HCC priority scoring system for ranking HCC candidates on the waiting list[15]. The scoring system devised by Rana *et al*[16], in which the most significant risk factors were previous transplantation and life support before transplant, could accurately predict the three-month survival following LT. The study by Weismüller *et al*[17] found that age, pre-transplant creatinine and cholinesterase were predictors of one-year survival after LT. Schaubel *et al*[18] evaluated a benefit-based survival system for allocating deceased-donor livers to chronic liver failure patients. They recommended that the proposed score based on the difference in five-year predicted mean lifetime should be used for guiding liver allocation. All the models discussed above are built on the data obtained from the entire population of recipients or patients with benign end-stage liver disease without considering HCC patients independently. To construct a prediction model for HCC patients usually requires a long period of

follow-up work, as HCC patients may survive for a time even with tumor recurrence. An analysis of rank correlations between benefit scores using different follow-up time points showed that the favorable time point should be three years or more[18]. The 2010 International Consensus Conference on LT for HCC accepted five years as the time point for survival assessment[5]. Thus, all living recipients in our study were followed for at least six years after LT, taking full account of the influence of tumor recurrence on long-term survival.

The UCSF criteria and the Fudan criteria are characteristic of a homogeneous extension of the Milan criteria boundary. Therefore, MHCAT based on these three criteria showed identical risk factors, such as AFP level, intraoperative blood loss and retransplantation. The Hangzhou criteria are somewhat different from the above three criteria when AFP is included. However, intraoperative blood loss and retransplantation were still significant predictors in MHCAT based on the Hangzhou criteria. The current HCC LT criteria focus more on morphological rather than biological factors. The other prognostic factors in MHCAT may reflect the biological characteristics of HCC. A number of studies on hepatectomy revealed that intraoperative blood loss was a predictive factor of HCC recurrence and cancer-related death[19-21]. However, the mechanism of the relationship between excessive blood loss and poor oncological outcomes has not been clearly identified. Potential reasons included tumor spillage and hematogenous spread during surgery, hypoperfusion and impaired oxygen delivery to vital organs and the introduction of some cytokines due to hemorrhagic shock. As the reason for retransplantation in our study was HCC recurrence, intraoperative blood loss and retransplantation in MHCAT may indicate the effect of circulating tumor cells (CTC) on HCC recurrence and metastasis. More intraoperative blood loss leads to an elevation in CTC level, whereas CTC homing in the graft may induce HCC recurrence, especially in the immunosuppressive state after LT. Therefore, CTC can serve as a potential icebreaker for HCC biological invasiveness.

In conclusion, we established a criteria-specific model for predicting long-term survival of HCC patients after LT, in which intraoperative blood loss, AFP level, retransplantation, TB, together with different indications for LT, may significantly affect the long-term survival of these recipients. The limitation of MHCAT lies in the data collected from our sole center, and this survival-prediction model may be statistically different among the four HCC LT criteria when it is applied in more centers. Therefore, MHCAT requires further evaluation in multicenter studies to optimize the current HCC LT criteria, which may facilitate pre-transplant clinical management, outcome prediction and decision-making.

ACKNOWLEDGMENTS

of the paper.

COMMENTS

Background
With a total number of liver transplantation (LT)s of more than 26000 cases, China is now second only to the United States. However, the five-year survival after LT in China is significantly lower than that in the United States and Europe, which is mainly responsible for the low survival rate of hepatocellular carcinoma (HCC) patients in China. Multicenter evaluations showed that allocation strategies and different regions could also affect long-term survival after LT. Therefore, it is of significant value to explore the risk factors for long-term survival of LT recipients with HCC in China.

Research frontiers
This study was conducted to build a model to predict long-term survival of HCC patients after LT with reference to different criteria and peri-transplant risk factors.

Innovations and breakthroughs
Currently, the HCC LT criteria are mainly based on tumor morphology, and less on biological characteristics. By combining all potential risk factors, model to predict long-term survival of hepatocellular carcinoma patients after liver transplantation (MHCAT) provides a more effective tool for survival prediction of HCC patients.

Applications
The MHCAT was built with reference to the four most representative HCC LT criteria, using accurate HCC patient data from a single center in China with a follow-up of at least six years. This model may help clinicians determine which candidates with HCC, especially in China, should receive LT.

Terminology
MHCAT refers to a criteria-specific long-term survival prediction model for hepatocellular carcinoma patients after liver transplantation, which was built using the Cox proportional hazards model with multivariate analysis.

Peer review
This manuscript describes a model to predict long-term survival of HCC patients after liver transplantation. The study design is scientifically sound and the findings are of potential clinical significance.

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EVIDENCE-BASED MEDICINE

Perinodular ductular reaction/epithelial cell adhesion molecule loss in small hepatic nodules

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Abstract

AIM: To investigate if loss of epithelial cell adhesion molecule (EpCAM) is associated with microinvasion in hepatocellular carcinomas (HCCs) in the presence of chronic hepatitis B.

METHODS: The expression of EpCAM, cytokeratin 7 (CK7) and CK19 in 112 hepatic nodules was studied, including 20 HCCs with nodules $\leqslant$ 3 cm, 26 HCCs with nodules > 3 cm, 20 high-grade dysplastic nodules, 26 cirrhotic, large regenerative nodules and 20 cases of cirrhosis.

RESULTS: Membranes of ductular reaction (DR) hepatobiliary cells, interlobular bile duct and some hepatic cells were positive for EpCAM expression. Active expression of DR/EpCAM was observed in the majority of noninvasive nodules (50/66, 75.76%); however, expression was absent in the major area of invasion in HCCs (42/46, 91.30%). DR/EpCAM loss in HCCs $\leqslant$ 3 cm was higher than in high-grade dysplastic nodules (HGDNs) (P < 0.05), cirrhotic, large regenerative nodules and cirrhosis (P < 0.01). Furthermore, patients (20 HCCs $\leqslant$ 3 cm, 26 HCCs > 3 cm, 20 HGDNs) with DR/EpCAM expression had a higher overall survival rate (P < 0.01) and lower early recurrence rate (P < 0.01). DR/EpCAM expression showed a close relationship with DR/CK7 and DR/CK19 expression (P < 0.01). The area under the receiver operating characteristic (ROC) curve of DR/EpCAM was similar to that of DR/CK7 and DR/CK19 (P > 0.05). The diagnostic specificity and diagnostic accuracy were both increased when DR/EpCAM, DR/CK7 and DR/CK19 were combined (P < 0.01).

CONCLUSION: DR/EpCAM loss may be a useful marker for determining microinvasion in HCCs $\leqslant$ 3 cm, but also for predicting prognosis.

Core tip: Epithelial cell adhesion molecule (EpCAM) may be a new marker of ductular reaction (DR) in routine pathology. We observed the morphological features of DR/EpCAM in 112 small hepatic nodules and compared

this with DR/cytokeratin 7 (CK7) and DR/cytokeratin 19 (CK19). The diagnostic value of DR/EpCAM was similar to DR/CK7 and DR/CK19; however, the diagnostic accuracy and specificity increased when these parameters were combined. Therefore, DR/EpCAM loss was confirmed to be a useful marker not only for determining microinvasion in HCCs ≤ 3 cm, but also for predicting prognosis.

INTRODUCTION

Distinguishing well-differentiated hepatocellular carcinomas (HCCs) from high-grade dysplastic nodules (HGDNs) or cirrhotic, large regenerative nodules (CLRNs) can be difficult in patients with small liver nodules. It has been suggested that invasion is a vital diagnostic feature of HCCs[1]. The appearance of microinvasion, however, particularly for the inexperienced liver pathologist without extensive exposure to resected hepatocellular nodules, may be similar to that of regenerative intraseptal hepatocyte buds. Consequently, new methods to identify invasion, particularly small foci of invasion, are required. Intraseptal hepatocyte buds are contiguous with ductular reactions (DRs) which are indicative of regeneration from intrabiliary progenitors[2,3]. DR is lost in the area of invasion in HCCs, but abundant in the majority of noninvasive nodules. It is hypothesized that DR immunostaining in small liver nodules may be a useful method for differential diagnosis. Recent studies of DR/cytokeratin 7 (CK7)[4] and DR/CK19[5,6], have supported this theory. DR can be expressed as CK7 or CK19; however, the diagnosis of small hepatic nodules remains a dilemma.

Epithelial cell adhesion molecule (EpCAM) is a cell surface protein expressed in normal epithelia, with the exception of squamous epithelia, epidermal keratinocytes, gastric parietal cells, myoepithelial cells, thymic cortical epithelium and hepatocytes[7,8]. Adult hepatocytes are EpCAM negative, with only bile duct epithelium being positive in liver tissue. During the regeneration and repair of liver tissues associated with focal nodular hyperplasia and cirrhosis, activation of EpCAM expression was observed, with high expression levels in so-called "ductular proliferations"[9]. The recent discovery of the expression of EpCAM in hepatocytes in the presence of chronic hepatitis B, indicates that these cells are a novel progeny of the hepatobiliary stem/progenitor cell compartment. Furthermore, transit amplifying DR hepatobiliary cells act as intermediates[4,10]. In other words, EpCAM-positive cells are associated with the differentiation of hepatocyte precursors, which are pres-

ent in the cirrhotic liver, dysplastic nodules or HCCs as tubular structures[11,12]. Consequently, it was hypothesized that EpCAM staining surrounding neoplastic nodules would not only be a marker for DR, but also a diagnostic method for invasion.

To investigate whether loss of DR/EpCAM is associated with invasion in HCCs in the presence of chronic hepatitis B and whether the expression of DR/EpCAM is superior to that of DR/CK7 and DR/CK19, a series of studies were performed to confirm the diagnostic value of DR/EpCAM.

MATERIALS AND METHODS

Case selection

The study was approved by the ethics committee of The Third Central Hospital of Tianjin Medical University. Written informed consent was obtained from each participant. As mentioned in the 2010 Barcelona Clinic Liver Cancer (BCLC) approach, it is crucial to make a diagnosis as early as possible for liver nodules ≤ 3 cm to achieve higher 5-year survival rates[13]. Small hepatic nodules (≤ 3 cm) diagnosed as HCCs, HGDNs or CLRNs following resection were selected from archival files. The size of the liver nodules was determined during surgical resection and small hepatic nodules were defined as a single tumor or 2 tumors (all < 3 cm). All patients with small hepatic nodules were diagnosed as having chronic hepatitis B, Child-Pugh A liver function and serum α-fetoprotein (AFP) < 400 ng/mL, and were followed up for a minimum of 24 mo. Patients with chronic hepatitis C, alcoholic hepatitis or autoimmune hepatitis and pathologically confirmed cholangiocarcinoma were excluded from the study. The specimens were selected from the Department of Pathology in our hospital. The HCCs with nodules > 3 cm and biopsy-proven cirrhosis (CIR) tissue on splenectomy for hypersplenism due to cirrhosis, were selected as the control groups. In total, 112 cases were assessed during HCC surveillance in hepatitis B virus-associated liver cirrhosis patients from Jan 1, 2005 to Feb 11, 2010, including 20 HCCs with nodules ≤ 3 cm, 26 HCCs with nodules > 3 cm, 20 HGDNs, 26 CLRNs and 20 cases of cirrhosis. Tumor recurrence was followed until patient death, or to the end of the study (Feb 1, 2013) using a serum AFP assay, chest radiography and ultrasound scanning or computed tomography every 3 mo after surgery. When recurrence was strongly suspected, selective hepatic angiography and ultrasound-guided biopsy were conducted for definitive diagnosis.

Evaluation of clinical pathology

The pathological features of all small liver nodules were evaluated by two senior pathologists blinded to patient clinical information. The criteria for HCC and HGDN diagnosis were according to the World Health Organisation and International Consensus Group for Hepatocellular Neoplasia guidelines[1,14]. According to the American Association for the Study of Liver Diseases guidelines[15] for the management of HCC, serum AFP, abdominal

Table 1 Postoperative follow-up of patients with hepatocellular carcinoma ≤ 3 cm, hepatocellular carcinoma with nodule > 3 cm and high-grade dysplastic nodules

Group	Follow-up time (mo)	Tumor-free survival time (mo)	Tumor-free survival rate		Early recurrence	Recurrence rate	Metastasis rate	Mortality rate
			1-yr	3-yr				
HCC > 3 cm	21.7	9.0	4/26	0/26	18/26	20/26	3/26	20/26
HCC ≤ 3 cm	60.1	35.5	18/20	7/20	7/20	14/20	3/20	1/20
HGDN	41.9	35.4	20/20	10/20	3/20	3/20	0/20	0/20

HCC: Hepatocellular carcinomas; HGDN: High-grade dysplastic nodules.

ultrasound examination and enhanced computed tomography (CT) or magnetic resonance imaging were used to diagnose HCC. Liver cirrhosis was diagnosed based on histological, serological and radiological tests. Small HCCs and HGDNs were accepted. Patients were followed up for a minimum of 24 mo (Table 1).

Immunohistochemistry

All samples were fixed with neutral 4% formaldehyde solution and 4 μm thick continuous sections were obtained for Hematoxylin and eosin (HE) staining, reticular fiber staining and CD34 staining for pathological diagnosis. The categorical diagnostic assignments for each of the hepatic nodules in this study were determined by consensus between 2-3 participating pathologists.

Immunohistochemical staining for EpCAM (VU1D9, Cell Signaling, United States; 1:500), CK7 and CK19 (OV-TL 12/30 and RCK108, Shanghai Biosun Sci & Tech Co., Ltd, Shanghai, China, Ready-to-Use) was performed according to the manufacturers' instructions. Briefly, 4 μm sections from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide for 15 min to inhibit endogenous peroxidase. Following heat-induced epitope retrieval in 0.1 mol/L of citrate buffer at pH 6.0 in a pressure cooker for 20 min, the slides were incubated with a mouse monoclonal antibody specific for each protein for 1 h at room temperature. Only CK7 received trypsinase-induced epitope retrieval. After incubation with a mouse anti-human secondary antibody, a reaction was performed using the EnVision plus detection system that contained biotin-free horseradish peroxidase-labeled polymers (Biosun Sci & Tech Co., Ltd). Staining was visualized using 3,39-diaminobenzidine substrate-chromogen (DAB) solution and counterstained with hematoxylin.

The DR of EpCAM, CK7, and CK19 was semiquantified as follows: the "-" label represents less than 25% DR positive cells (diffuse loss of DR); the label of "+/-" represents 26%-75% DR positive cells (focal loss of DR) and the label of "+" represents more than 76% DR positive cells (active DR). Cases were evaluated by independent reviewers along with 2 experienced observers.

Statistical analysis

The percentage of DR/EpCAM focal loss (+/-) and diffuse loss (-) in all HCCs represented the sensitivity, and the percentage of active DR in non-HCCs repre-

sented the specificity of immunostaining. The paired χ^2 test and Fisher's exact test were used for group comparisons. Pearson's correlation coefficient was used to determine the relationship between antibodies and clinical data. The receiver operating characteristic (ROC) curve was plotted for each biomarker. The area under the ROC curve (AUC) was calculated to compare the values of DR/EpCAM, CK7 and CK19 as diagnostic biomarkers. Traditionally, a poorly designed experiment has an AUC of 0.5, whereas a well-designed experiment (one that has zero false positives and zero false negatives) has an AUC of 1.0. A *Z* test was used to compare between the two groups. A follow-up comparison between the two groups was performed and analyzed with the independent-samples Student's *t* test. The tumor-free survival time was measured from the date of resection to the detection of recurrent tumor or the end point of this study. Recurrent tumor within two years after surgery was considered early recurrence. The survival curves were generated by the Kaplan-Meier method and compared by the log rank test. A *P* value of < 0.05 was considered statistically significant. A *u* test was used to compare two rates. Statistical analyses were performed using the SPSS software (Version 16.0; SPSS, Inc., Chicago, IL, United States).

RESULTS

Patients with small HCC nodules were classified as early stage BCLC, while patients with nodules > 3 cm were classified as intermediate or advanced BCLC. A total of 112 cases participated in the current study (20 HCCs ≤ 3 cm, 26 HCCs > 3 cm, 20 HGNs, 26 CLRNs and 20 CIRs).

The mean age of the 73 male and 39 female patients was 52.68 years. Patients were followed up for 3 to 90 mo. The follow-up time was shorter than 1 year in patients with HCCs due to mortality. A total of 18 patients died, including 1 who died of causes unrelated to HCC or cirrhosis. The death rate, 1-year and 3-year tumor-free survival rate, were significantly different between patients with HCCs ≤ 3 cm and HCCs > 3 cm (*P* < 0.01), and the early recurrence rate was also significantly different between the two groups (*P* < 0.05). Only the recurrence rate was significantly different between patients with HCCs ≤ 3 cm and HGDNs (*P* < 0.01). Twenty-six cases of CLRN and 20 cases of cirrhosis were followed up for at least 24 mo (24-77 mo, mean 45.59 mo), and
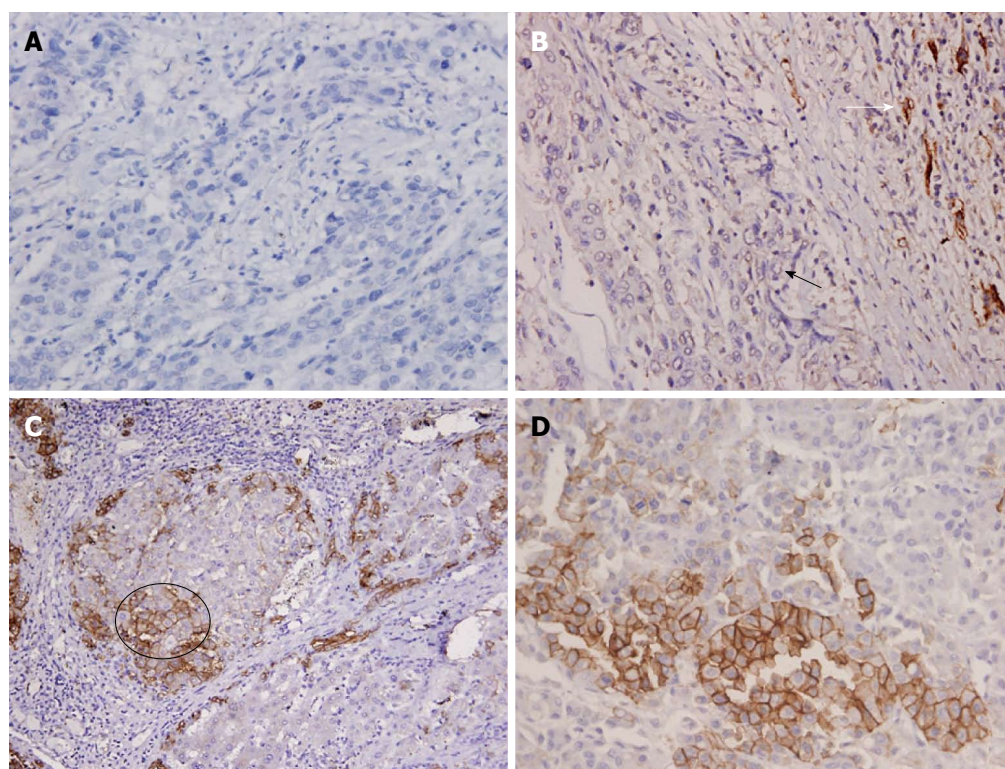
Figure 1 Immunohistochemistry staining pictures. Diffuse loss of DR/EpCAM in HCC > 3 cm (A, IHC, × 400), a surgical sample of HCC ≤ 3 cm (a moderately differentiated HCC), and the area of foci invasion (black arrow) in HCC ≤ 3 cm; the invasive area showed diffuse loss of DR/EpCAM; but positive on the biliary cells between hepatic lobules (white arrow) (B, IHC, × 400). Active DR/EpCAM was observed in a cirrhotic nodule (C, IHC, × 200), and part of liver cells were EpCAM positive (circle). DR/EpCAM was focally positive (D, IHC, × 400), and the cells showed features of hepatocyte inside the nodules of HCCs. HCC: Hepatocellular carcinoma; DR: Ductular reaction; EpCAM: Epithelial cell adhesion molecule; IHC: Immunohistochemistry.

no malignancy was observed.

DR/EpCAM immunohistochemical pattern

EpCAM-immunoreactive DRs were analyzed at the epithelial-stromal boundaries between the neoplastic tissue and/or paraneoplastic tissue of each nodule. In this study, EpCAM staining was positive on the membrane of hepatic cells and biliary cells. There was diffuse loss of DR/EpCAM in HCCs > 3 cm (Figure 1A). In the HCC ≤ 3 cm group, there were twelve well differentiated HCCs (Figure 2A) and eight moderately differentiated HCCs (Figure 1B). The expression of DR was negative around the HCCs ≤ 3 cm (Figures 1B, 2A, 3), but positive between the dysplastic nodules (Figure 2B, C) and cirrhotic nodules (Figure 1C). A significant number of HCCs were positive for EpCAM (Figure 1D) and some liver cells were EpCAM positive (Figure 1C, circle). These cells showed spotted or focal staining; however, the positive cells were located inside neoplastic cells and not in the boundaries between neoplastic cells.

DR/EpCAM in different clinical groups

Diffuse loss or focal loss of DR/EpCAM was evident in most HCCs ≤ 3 cm and in HCCs with nodules > 3 cm (42/46, 91.30%). However, only 8, 5 and 3 cases presented with diffuse loss or focal loss of DR/EpCAM in HGDNs, CLRNs and CIRs, respectively. Therefore, the positive rate of active DR/EpCAM was 75.76% (50/56)

in non-invasive hepatic nodules. DR/EpCAM staining was significantly different between HCCs ≤ 3 cm and HGDNs ($P < 0.05$), HCCs ≤ 3 cm and CLRNs or CIRs ($P < 0.01$). Specimens from HCCs ≤ 3 cm showed greater DR/CK19 loss than specimens from HGDNs, CLRNs and CIRs (all $P < 0.01$). DR/CK7 loss in HCCs ≤ 3 cm was less than that in HCCs with nodules > 3 cm ($P < 0.05$), and more than CLRNs and CIRs (both $P < 0.01$). The distribution of DR among the different groups is listed in Table 2.

DR/EpCAM expression compared with DR/CK7 and DR/CK19 expression

Semiquantitative analysis of DR/EpCAM expression showed a significant correlation between DR/CK7 and DR/CK19 ($P < 0.01$). The sensitivity of DR/EpCAM loss, DR/CK7 loss and DR/CK19 loss in all HCCs (HCCs ≤ 3 cm and HCCs > 3 cm) was 91.30%, 78.26%, and 89.13%, respectively, and the specificity was 75.76%, 80.30%, and 77.27%, respectively. The ROC curve showed that the area under the ROC curves of DR/EpCAM loss (0.864) was similar to DR/CK7 loss (0.727), DR/CK19 loss (0.831) and active GPC3 (0.914) (Z = 1.51, 0.41, and 0.69, respectively; $P > 0.05$). The diagnostic accuracy of the loss of DR/EpCAM, DR/CK7 and DR/CK19 was 82.14%, 79.46% and 82.14%, respectively, and the negative predictive value (NPV) was 94.20%, 84.13% and 91.07%, respectively. The results were then
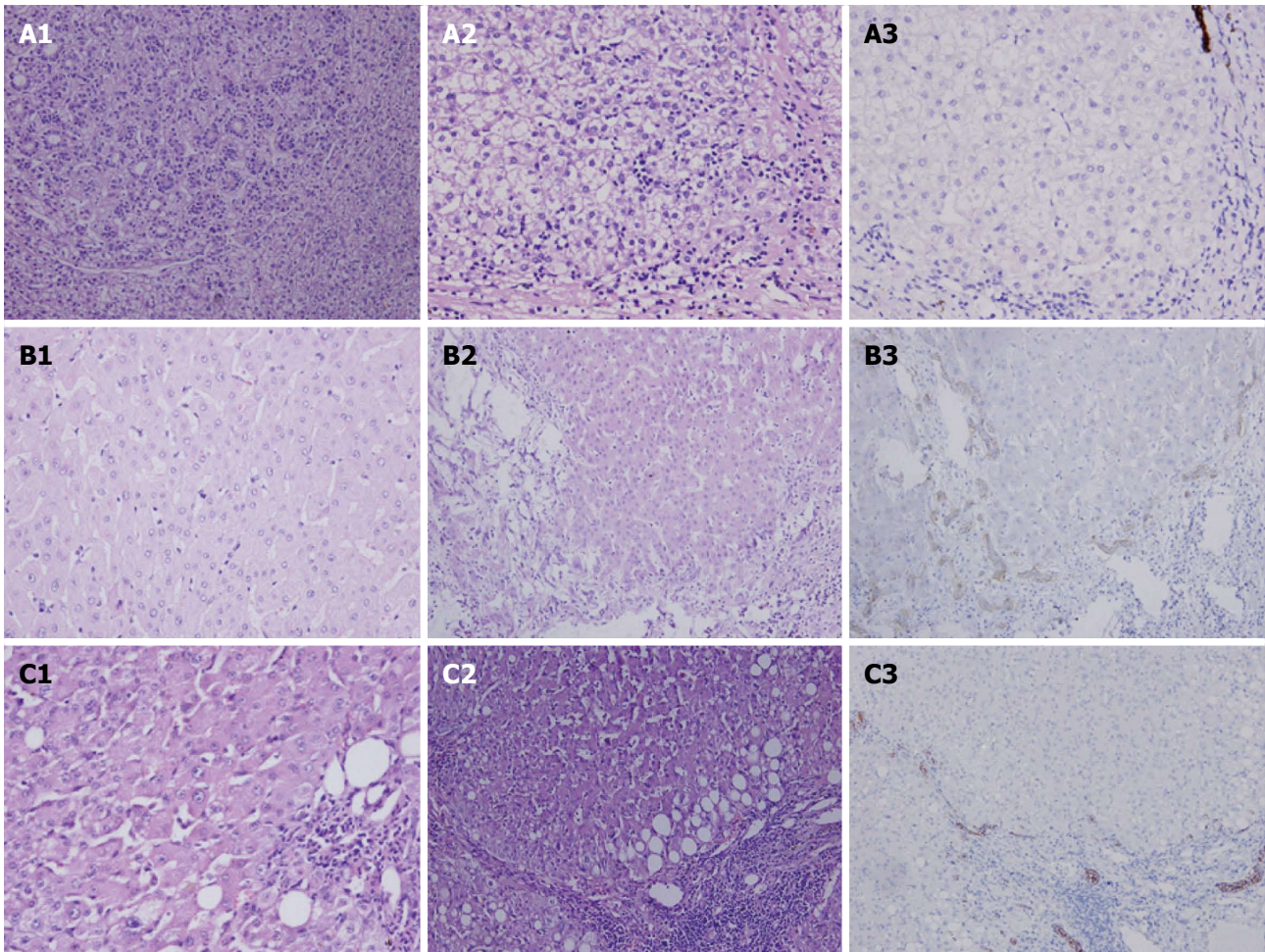
Figure 2 Immunohistochemistry and hematoxylin-eosin staining pictures. A surgical sample of HCC ≤ 3 cm (a well-differentiated HCC), with pseudogland-like structure (A1, HE, × 200), and the features of the boundary area of the same nodule (A2, HE, × 200); this area showed diffuse loss of DR/EpCAM (A3, IHC, × 200). HGDN had an increased cell density, more than 1.5 times higher than the surrounding non-tumoral liver, often with an irregular trabecular pattern (2-3 cells thick), and small cell dysplasia (B1, HE, × 400). The cirrhotic, large regenerative nodule (CLRN) showed a mild increase in cell density with a monotonous pattern, without cytologic atypia, although they may have large cell dysplasia (C1, HE, × 400). The features of the boundary area of HGDN (B2, HE, × 200) and CLRN (C2, HE, × 200) with focal fatty change are shown respectively. Active DR/EpCAM was present in a high-grade dysplastic nodule (B3, IHC, × 200) and a CLRN (C3, IHC, × 200). HCC: Hepatocellular carcinoma; HGDN: High-grade dysplastic nodule; CLRN: Cirrhotic, large regenerative nodule; DR: Ductular reaction; EpCAM: Epithelial cell adhesion molecule; IHC: Immunohistochemistry; HE: Hematoxylin-eosin staining.

Table 2 Ductular reaction distribution among different groups

Group	DR/EpCAM			DR/CK7			DR/CK19		
	-	+/-	+	-	+/-	+	-	+/-	+
HCC1	17	8	1	13	11	2	16	9	1
HCC2	11	6	3	7	5	8	9	7	4
HGDN	1	7	12	2	5	13	1	5	14
CLRN	0	5	21	0	4	22	0	5	21
CIR	0	3	17	0	2	18	0	4	16

HCC1: HCC > 3 cm; HCC2: HCC ≤ 3 cm. HCC: Hepatocellular carcinoma; HGDN: High-grade dysplastic nodules; CLRN: Cirrhotic large regenerative nodule; CIR: Cirrhosis; DR: Ductular reaction; CK: Cytokeratin.

combined into a new group. If 2 or more results were positive for DR/EpCAM, DR/CK7 and DR/CK19, then the tumor was considered to be DR positive. If two or more results showed focal/diffuse loss of DR/Ep-CAM, DR/CK7 or DR/CK19, the tumor was considered to be DR negative. Using this method, the sensitivity was 91.30%, but the specificity increased to 98.48%. The specificity of the new group was higher than that for DR/EpCAM, DR/CK7 or DR/CK19 (u = 3.90, 3.73, and 3.39 respectively; $P < 0.01$). The area under the ROC curves of the combined group was 0.924 and was similar to DR/EpCAM and DR/CK19 (Z = 1.23 and
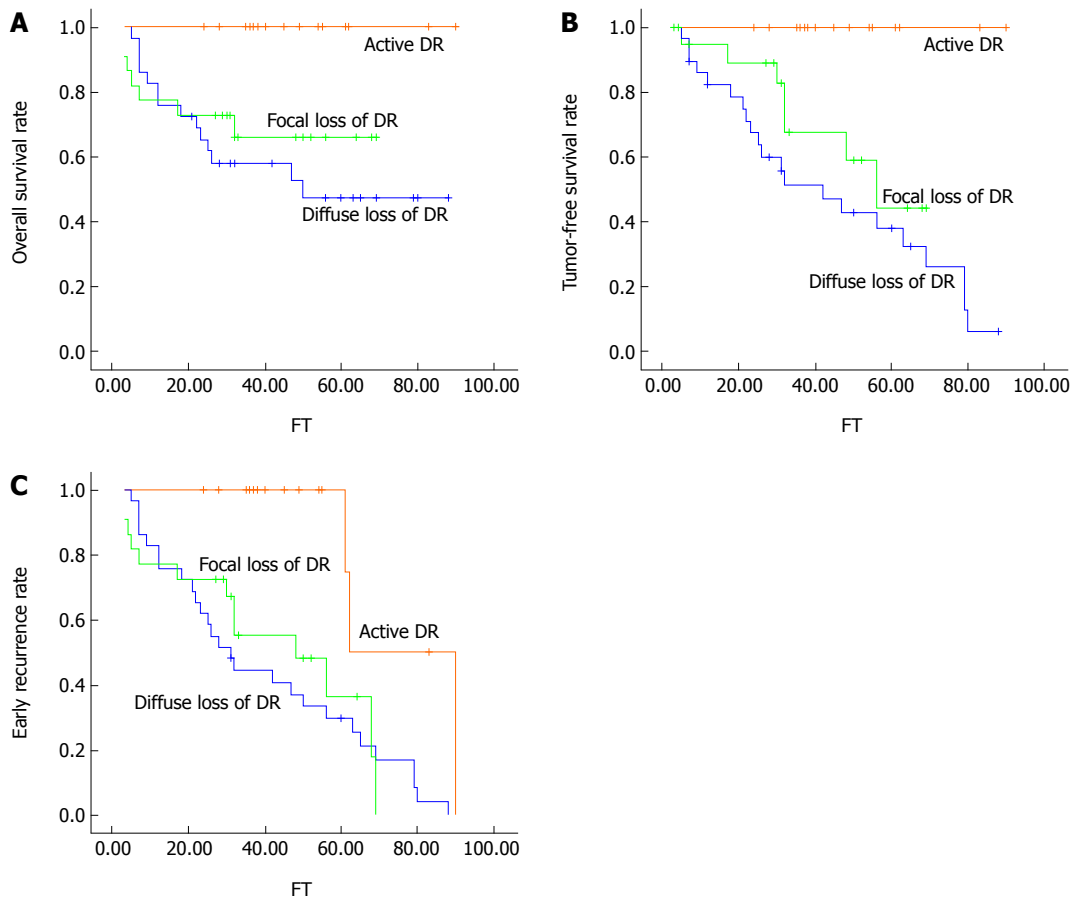
Figure 3 Survival analysis of patients with hepatocellular carcinoma ≤ 3 cm (A) and hepatocellular carcinoma with nodules > 3 cm (B) or high-grade dysplastic nodules (C). DR: Ductular reaction; FT: Follow-up time.

1.88, respectively; $P > 0.05$), but significantly higher than DR/CK7 ($Z = 3.36$, $P < 0.01$). The diagnostic accuracy of this method was 95.54% and the NPV was 94.20%. The diagnostic accuracy was increased by this method ($u = 3.18$, 3.64, and 3.18, respectively, $P < 0.01$), but the NPV was similar to DR/EpCAM and DR/CK19 ($u = 0.06$, and 0.90, $P > 0.05$) and higher than DR/CK7 ($u = 2.42$, $P > 0.05$).

DR/EpCAM during follow-up

Of 66 patients (20 HCCs ≤ 3 cm, 26 HCCs > 3 cm, 20 HGDNs), 37 (16 DR/EpCAM positive and 21 DR/EpCAM negative) exhibited tumor recurrence during the follow-up period. Thirty-two patients had intrahepatic tumor recurrence only, while 6 had extrahepatic metastasis. Of 16 patients with DR/EpCAM positive nodules, none showed early recurrence. Of 50 patients with DR/EpCAM negative nodules, the overall survival rate and tumor-free survival rate were significantly lower than those with DR/EpCAM positive nodules (overall survival rate: $\chi^2 = 8.285$, $P = 0.004$; tumor-free survival rate: $\chi^2 = 14.400$, $P = 0.000$) ($P < 0.01$) (Figure 3A, B). The incidence of early recurrence in patients with DR/EpCAM negative nodules was significantly higher than in those with DR/EpCAM positive nodules ($\chi^2 = 10.773$, $P = 0.001$) ($P < 0.01$) (Figure 3C).

DISCUSSION

The most proximal branches of the biliary tree (*i.e.*, the canals of Hering and ductules) comprise, or at least harbor, facultative hepatic stem cells[2,3]. These intraseptal hepatocytes most likely represent buds of newly formed hepatocytes arising from branches of the biliary tree[3]. It was previously demonstrated that DR is a sign of newly regenerating hepatocytes in chronic hepatitis B, and therefore did not develop from either biliary metaplasia of malignant hepatocytes, or from the outgrowth of biliary cells[4]. The absence of DR is a useful marker for characterizing the areas of microinvasion in HCCs ≤ 3 cm[4,5], especially for HCCs in the presence of chronic hepatitis B cirrhosis. Based on this understanding, the loss of DR/EpCAM is also helpful in defining microinvasion and distinguishing HCC ≤ 3 cm from other hepatic nodules.

The results from the current study confirm that DR/EpCAM underwent focal or diffuse loss in the majority of invasive hepatocellular nodules (HCCs ≤ 3 cm and HCCs > 3 cm), showing an overtly invasive phenotype. The loss of DR/EpCAM was associated with the areas of morphologically identified microinvasion, whereas noninvasive areas showed abundant DR/EpCAM expression at hepatocellular-stromal boundaries in the

same tissue section. In contrast, DR/EpCAM expression was evident in the majority of noninvasive nodules, such as HGDNs, CLRNs and cirrhotic nodules. The degree of DR/EpCAM loss differed between HCCs ≤ 3 cm and HGDNs, indicating that DR/EpCAM may be absent in the small foci of invasive areas around cancerous nodules. The absence of DR/EpCAM in the foci of invasion suggests that immunostaining for these structures may be a useful diagnostic tool and may assist pathologists in identifying the appearance of the histologic lesion. These results are in accord with the study of CK19 expression. Perinodular CK19 loss was consistently observed in HCCs and the altered expression of CK19 in cirrhotic nodules, dysplastic nodules and HCCs was an underlying mechanism for the reproducible extralesional CK19 pattern that paralleled progressive stages of intranodular hepatocarcinogenesis[5]. Results from the current study showed that EpCAM was expressed in both proliferating bile ducts and interlobular bile ducts. A considerable overlap between DR/EpCAM and both DR/CK7 and DR/CK19 was observed. However, when DR/EpCAM was combined with DR/CK7 and DR/CK19, the diagnostic accuracy and diagnostic specificity were significantly increased.

EpCAM was originally identified as a marker of carcinoma, attributable to its high expression in rapidly proliferating tumors of epithelial origin[16]. EpCAM positive HCCs were a subset of cells with cancer stem cell features[12], which was similar to CK19[17]. However, the positive expression of EpCAM and CK19 in tumor cells was low in HCCs[5,12]. Therefore, only the morphological features of EpCAM, CK7 and CK19 positive HCCs were observed, and these cells showed spotted or focal staining in neoplastic cells, but not in the boundaries between neoplastic cells. The morphological features and the distribution of these positive cells were different from the cells of DR. This characteristic of HCCs did not result in an adverse effect on the role of DR as a marker of EpCAM, CK7 and CK19.

Moreover, patients with active DR/EpCAM nodules (in HGDNs, HCCs ≤ 3 cm and HCCs > 3 cm) had a better prognosis, including a higher overall survival rate, 1-year and 3-year tumor-free survival rate, and lower early recurrence rate. Consequently, the loss of DR/EpCAM had a close relationship with invasive HCCs and predicted an increased incidence of recurrence, regardless of HCCs ≤ 3 cm or HCCs > 3 cm.

In conclusion, DR loss is an important feature of the epithelial-stromal compartment in the malignant progression of HCCs with cirrhosis. DR/EpCAM expression may be used as a diagnostic marker. The histological pattern of stromal invasion and altered expression of DR/EpCAM at epithelial-stromal boundaries was determined by DR immunostaining. In particular, the differential diagnosis of HCCs ≤ 3 cm and HGDNs may improve diagnostic confidence in pathologists faced with the spectrum of lesions which occur in small hepatocellular nodules. In addition, the loss of DR/EpCAM is associated with increased invasiveness of HCC and poor prognosis.

ACKNOWLEDGMENTS

COMMENTS

Background

Distinguishing a well-differentiated hepatocellular carcinoma (HCC) from high-grade dysplastic nodules (HGDNs) or cirrhotic, large regenerative nodules can be difficult in patients with small liver nodules. It has been suggested that invasion is a vital diagnostic feature of HCC. However, the appearance of microinvasion, particularly for the inexperienced liver pathologist without extensive exposure to resected hepatocellular nodules, may be similar to that of regenerative intraseptal hepatocyte buds. Thus, new methods to identify invasion, particularly small foci of invasion, are required. These intraseptal hepatocyte buds are contiguous with ductular reactions (DRs) which are indicative of regeneration from intrabiliary progenitors. DR is lost in the area of invasion in HCCs, whilst abundant in the majority of noninvasive nodules. It is hypothesized that DR immunostaining in small liver nodules may be a useful method for differential diagnosis. Recent studies of DR/cytokeratin 7 (CK7) and DR/CK19, have supported this theory. DR can be expressed as CK7 or CK19, however, the diagnosis of small hepatic nodules remains a dilemma.

Research frontiers

Epithelial cell adhesion molecule (EpCAM) positive cells are associated with the differentiation of hepatocyte precursors, which are present in the cirrhotic liver, dysplastic nodules or HCCs as tubular structures. Consequently, it was hypothesized that EpCAM staining surrounding the neoplastic nodules would not only be a marker for DR, but also a diagnostic method for invasion recognition.

Innovations and breakthroughs

The authors observed the morphological features of DR/EpCAM in 112 small hepatic nodules and compared these with DR/CK7 and DR/CK19. It has been proved that the diagnostic value of DR/EpCAM was similar to DR/CK7 and DR/CK19, but the diagnostic specificity was increased by the combination of DR/CK7 and DR/CK19. Furthermore, DR/EpCAM loss may predict poor prognosis.

Applications

This study provides new knowledge on the differential diagnosis of small liver nodules and may be useful for daily routine work in pathology. The study results suggest that DR/EpCAM loss may be a new useful marker not only for recognizing microinvasion in small HCCs, but also for differentiating HCCs with nodules ≤ 3 cm from HGDNs.

Peer review

This is a very interesting report. A lot of hard work was done and new information was added to our knowledge. The paper was written very well and provided a set practical procedure for daily routine work. This study can be a guiding method for the differential diagnosis among different liver nodular lesions with their immunohistochemical features.

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CASE CONTROL STUDY

Serum beta 2-microglobulin as a biomarker in inflammatory bowel disease

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Abstract

AIM: To investigate the diagnostic utility of beta 2 microglobulin (B2-M) levels and analyze this correlation with the activity of inflammatory bowel disease (IBD).

METHODS: Overall, 78 IBD patients and 30 healthy controls were enrolled in the study. We examined B2-M serum levels in 43 ulcerative colitis (UC) patients, 35 with Crohn's disease (CD) and 30 control subjects, using an enzymatic method. Patients were divided into two groups according to two disease types: active and in remission. Subjects were also divided into two subgroups according to extent of the disease: left-side and pancolitis for UC and ileitis and ileocolitis for CD. All groups were compared for mean serum B2-M levels and also examined to see whether there was a correlation between serum B2-M levels and other inflammatory markers.

RESULTS: The mean serum B2-M levels in the control group, UC and CD were 1.71, 2.41 and 2.24 respectively. B2-M values $\geq$ 1.96 mg/L had a 62% sensitivity, 76% specificity, a 79% positive predictive value, and a 58% negative predictive value for UC patients. B2-M values $\geq$ 1.70 mg/L had 80% sensitivity, 53% specificity, 66% positive predictive value, and 69% negative predictive value for CD patients. Mean B2-M values were significantly higher in ulcerative colitis and Crohn's disease patients than in healthy controls (UC 2.41 ± 0.87 *vs* 1.71 ± 0.44, *P* = 0.002; CD 2.24 ± 1.01 *vs* 1.71 ± 0.44, *P* = 0.033). Also, mean B2-M values were significantly higher in active disease when compared to patients in remission (UC 2.66 ± 0.92 *vs* 1.88 ± 0.41, *P* = 0.004; CD 2.50 ± 1.15 *vs* 1.73 ± 0.31, *P* = 0.033). The difference between groups (UC and CD) in terms of serum B2-M levels was statistically insignificant (2.41 ± 0.87 *vs* 2.24 ± 1.01, *P* > 0.05 respectively).

CONCLUSION: Serum B2-M levels may be used as an activity parameter in IBD.

Core tip: Endoscopy has been the gold standard for diagnosing and following patients with inflammatory bowel disease (IBD). However, it is still an expensive and invasive method. Beta 2 microglobulin levels of intestinal inflammation represent an easy, non-invasive, cheap and objective diagnostic biomarker for active IBD.

INTRODUCTION

Inflammatory bowel diseases (IBD) are characterized by idiopathic and chronic inflammation of the intestinal

tract and consist of ulcerative colitis (UC), Crohn's disease (CD) and indeterminate colitis. Disease activity in IBD is determined using both direct and non-invasive laboratory markers. However, endoscopic examination is still the gold-standard diagnostic test, even though it is invasive and expensive[1,2]. Laboratory markers such as C reactive protein (CRP), erythrocyte sedimentation rate (ESR), white blood count (WBC) and platelet count, albumin, fecal calprotectin and acid glycoprotein (orosomucoid) have been investigated in IBD with different aims including diagnosis, disease activity, response to therapy, and estimate of relapse with a wide range of sensitivity and specificity[3-6]. Unfortunately, an ideal marker for IBD that is easy, cheap, rapid to perform, disease specific, and having prognostic merit against relapse or recurrence of the disease has not yet been identified[7].

Beta 2 microglobulin (B2-M) is a low-molecular-weight protein released by activated T and B lymphocytes. The estimated half-lifetime is short (2 h)[8]. B2-M has been shown to increase in several inflammatory and hematologic disorders, such as systemic lupus erythematosus (SLE), acquired immunodeficiency syndrome, multiple myeloma, lymphoma and leukemia[9-12]. To our knowledge, only a few studies have investigated B2-M in IBD, and the results are conflicting[13-16]. The aim of the present study is to evaluate B2-M levels in patients with UC and CD and to compare their validity with the other tests used in clinical practice.

MATERIALS AND METHODS

Patients
A total of 108 subjects were included in the study. Seventy-eight patients had IBD (43 UC and 35 CD patients). The diagnosis of IBD was based on standard clinical, radiological, endoscopic and histological criteria. All enrolled IBD patients had normal renal function and had no other disease that could influence serum levels of B2-M.

IBD patients and the control group were tested for complete blood count, ESR, CRP and albumin at the time of entry. All IBD patients underwent a total colonoscopic examination. The terminal ileum was also examined in patients with CD. All IBD patients were divided into two groups according to disease activity: either active or in remission. Patients were also divided into two subgroups according to the extent of the disease: left and extensive for UC, or ileitis and ileocolitis for CD. On the initial examination, two patients had proctitis and these were included in the left-sided group for comparison. All groups were compared for mean serum B2M levels.

The extent of UC was classified according to the Montreal classification[17]. Involvement up to the splenic flexure was defined as left-sided colitis, and disease extension to the proximal part of the splenic flexura was defined as extensive UC. Clinical disease activity was evaluated using a modified Truelove-Witts severity index (MTWSI). Clinically active disease was defined as having an estimated MTWSI score of 4 or higher; patients with a score lower than 4 were considered to be in remission (inactive)[18]. The disease activities of CD patients were classified according to the Harvey-Bradshaw index[19].

B2M assay
In the ADVIA 2400 Chemistry B2-M assay, a sample is diluted and reacted with a buffer that contains latex particles coated with an antibody specific for B2-M. The formation of the antibody-antigen complex during the reaction results in an increase in turbidity, the extent of which is measured as the amount of light adsorbed at 545 nm. The B2-M concentration in a sample is determined by constructing a standard curve from the absorbance of a reagent blank and a single-level calibrator. Blood samples were collected from a peripheral vein after an overnight fast and were subjected to centrifugation at the speed of 3000 revolutions per minute for 10 min at 4 ℃, to obtain serum. All blood samples were stored at -20 ℃ immediately after separation from peripheral blood prior to analysis.

Statistical analysis
Data analysis was performed using the Statistical Package for Social Sciences (SPSS) version 13 software (SPSS Inc., Chicago, IL, United States). Values are presented in the study as mean ± SD. Continuous variables were analyzed using unpaired Student t tests or a 1-way analysis of variance. χ^2 analysis was used for categorical variables. The Pearson correlation coefficient was utilized to analyze the correlation between B2-M and other markers. The sensitivity and specificity of B2-M, CRP, ESR and WBC levels for the evaluation of patients were calculated with various cut-off ranges, and the receiver operating characteristic (ROC) curves were drawn. A "P" value of less than 0.05 was considered statistically significant.

RESULTS
The demographic and clinical characteristics of patients and control subjects are summarized in Table 1. Gender and age were comparable for IBD patients and the control group. The mean serum B2-M levels in the control group, UC and CD were 1.71, 2.41, and 2.24 respectively. Mean B2-M and ESR values were significantly higher in UC and CD patients than in healthy controls. The difference between groups (UC and CD) in terms of serum B2-M, ESR and albumin levels was statistically insignificant. Mean albumin and hemoglobin values were significantly lower in UC and CD patients than controls. Mean CRP and WBC levels were statistically insignificant in comparing patients and controls (Table 1). No correlation was found between B2-M and other inflammation markers for UC patients (CRP: $r = 0.281$, $P = 0.079$, ESR: $r = 0.14$, $P = 0.383$, WBC: $r = 0.222$, $P = 0.162$). However, there was a correlation for B2-M with CRP and ESR for CD patients (CRP: $r = 0.79$, $P = 0.001$, ESR: $r = 0.76$, $P = 0.001$).

Table 1 Demographic characteristics and comparison of serum Beta 2 microglobulin levels with other laboratory markers between patients and controls *n* (%)

	Controls	UC	CD	*P* value	
	n = 30	*n* = 43	*n* = 35		
Mean age	38.90 ± 11.05	42.72 ± 13.52	38.28 ± 13.15	0.254	
Gender (F/M)	18 (60)/12 (40)	17 (40)/26 (60)	19 (54)/16 (46)	0.188	
Duration (yr)		5.04 ± 5.81	4.50 ± 3.70	0.729	
Inactive/active		14 (33)/29 (67)	12 (34)/23 (66)		
		Left type 29 (67)	Ileal type 24 (69)		
Disease location		Extensive 14 (33)	Ileocolonic 11 (31)		
B2-M	1.71 ± 0.44	2.41 ± 0.87	2.24 ± 1.01	0.002	UC *vs* C 0.002
					CD *vs* C 0.033
					UC *vs* CD 0.642
CRP	0.57 ± 0.83	1.82 ± 2.94	1.59 ± 2.79	0.188	
ESR	10.18 ± 11.31	21.21 ± 18.37	22.76 ± 22.81	0.021	UC *vs* C 0.047
					CD *vs* C 0.026
					UC *vs* CD 0.931
WBC ($\times 10^3$)	7.09 ± 1.75	7.52 ± 2.84	7.48 ± 3.91	0.190	
Albumin	4.43 ± 0.24	4.00 ± 0.62	4.05 ± 0.63	0.005	UC *vs* C 0.006
					CD *vs* C 0.023
					UC *vs* CD 0.916

Data are expressed as absolute numbers (percentage), mean ± SD or median (interquartile range). UC : Ulcerative colitis; CD : Crohn Disease; F: Female; M: Male; B2-M: Beta 2 microglobulin; CRP: C reactive protein; WBC: White blood count; C: Control; ESR: Erythrocyte sedimentation rate.

Table 2 Overall accuracy and receiver operating characteristic analyses of beta 2 microglobulin and other inflammation markers to differentiate active from inactive ulcerative colitis

	AUC	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)
B2-M (cut off: 2.02)	0.757	79.3	78.6	88.5	64.7
CRP (cut off: 0.55)	0.731	67.9	75.0	86.4	50.0
WBC ($\times 10^3$) (cut off: 6.50)	0.656	72.4	58.3	80.8	46.7
ESR (cut off: 14.5)	0.677	69.0	58.3	80.0	43.8

AUC: Area under the curve; B2-M: Beta 2 microglobulin; CRP: C reactive protein; WBC: White blood count; ESR: Erythrocyte sedimentation rate; NPV: Negative predictive value; PPV: Positive predictive value.

Table 3 Comparison of serum beta 2 microglobulin levels with other laboratory markers between active and inactive ulcerative colitis patients

	Inactive CD	Active CD	*P* value
	n = 14	*n* = 29	
B2-M	1.88 ± 0.41	2.66 ± 0.92	0.004
CRP	0.41 ± 0.27	2.43 ± 3.34	0.046
ESR	14.00 ± 7.85	24.20 ± 20.65	0.106
WBC ($\times 10^3$)	6.41 ± 1.47	7.96 ± 3.16	0.119
Albumin	3.99 ± 0.50	4.01 ± 0.67	0.948

B2-M: Beta 2 microglobulin; CRP: C reactive protein; WBC: White blood count; ESR: Erythrocyte sedimentation rate.

Table 4 Comparison of serum beta 2 microglobulin levels with other laboratory markers between active and inactive Crohn's disease patients

	Inactive CD	active CD	*P* value
	n = 12	*n* = 23	
B2-M	1.73 ± 0.31	2.50 ± 1.15	0.033
CRP	0.24 ± 0.12	2.23 ± 3.22	0.050
ESR	11.45 ± 11.21	28.17 ± 25.08	0.044
WBC ($\times 10^3$)	7.81 ± 2.28	7.33 ± 4.53	0.742
Albumin	4.50 ± 0.42	3.83 ± 0.60	0.002

CD: Crohn's disease; B2-M: Beta 2 microglobulin: CRP: C reactive protein; ESR: Erythrocyte sedimentation rate; WBC: White blood count.

Ulcerative colitis

B2-M values ≥ 1.96 mg/L had a 62% sensitivity, 76% specificity, a 79% positive predictive value (PPV), and a 58% negative predictive value (NPV) for UC patients. ROC curve analysis suggested that the optimum B2-M cut-off point for active UC was 2.02 mg/L, with a sensitivity, specificity, PPV, and NPV of 79%, 78%, 88%, and 64% respectively (Table 2). The same analyses for other inflammation markers are summarized in Table 2. Serum B2-M and CRP levels of the active UC patients were significantly higher than those of inactive patients (Table 3). There was not a significant correlation between B2-M levels and other inflammatory markers in patients with UC. Fourteen patients were classified as having pancolitis and 29 patients as having left-sided pancolitis, according to endoscopic examination upon study entry. There was no significant difference between B2-M and UC extension (*P* = 0.694).

Table 5 Overall accuracy and receiver operating characteristic analyses of beta 2 microglobulin and other inflammation markers between active and inactive ulcerative colitis patients

	AUC	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)
B2-M (Cut off: 1.84)	0.828	78.3	75.0	85.7	64.3
CRP (Cut off: 0.35)	0.903	78.3	81.8	90.0	64.3
WBC ($\times 10^3$)	0.379	47.8	56.5	68.8	33.3
(Cut off: 6.80)					
ESR (Cut off: 13.5)	0.761	78.3	72.7	85.7	61.5

AUC: Area under the curve; B2-M: Beta 2 microglobulin; CRP: C reactive protein; WBC: White blood count; ESR: Erythrocyte sedimentation rate; NPV: Negative predictive value; PPV: Positive predictive value.

Crohn's disease

B2-M values $\geq$ 1.70 mg/L had 80% sensitivity, 53% specificity, 66% PPV, and 69% NPV for CD patients. Serum B2-M, CRP, and ESR levels of the active CD patients were significantly higher than those of inactive patients (Table 4). ROC curve analysis suggested that the optimum B2-M cutoff point for active CD was 1.84 mg/L, with a sensitivity, specificity, PPV, and NPV of 78%, 75%, 86%, and 64% respectively (Table 5). B2-M levels were correlated with CRP, ESR, PLT, and age in patients with CD, but not with WBC and disease duration. Twelve patients were classified with the ileal type and 23 patients with the ileocolonic type, according to endoscopic examination upon study entry. There was no significant difference between B2-M and CD location (P = 0.165).

DISCUSSION

The clinical courses of UC and CD are characterized by exacerbations and remissions, which occur spontaneously or in response to medical treatment[20-23]. Disease flares occur in an indiscriminate way and are mostly unpredictable. Inflammatory markers have been investigated in IBD for diagnosis, disease activity and prediction of relapse. As an inflammatory marker, B2-M has been investigated in patients with IBD in several studies. However, the results were conflicting and not all authors were able to confirm a correlation.

Among the laboratory markers used in IBD practice, CRP is the most studied and the most popular one. It is accepted as a good predictor of disease activity in IBD. However, the CRP assay correlates better for CD than for UC[4,24,25]. Other laboratory markers, including WBC, platelets, albumin, sialic acid, orosomucoid, fibrinogen, lactoferrin and serum amyloid have variable associations with the disease activity of IBD. Similarly, we also detected a positive correlation between CRP and disease activity in the present study.

A recent study by Zissis *et al*[13] investigated B2-M serum levels in 87 UC patients, 74 with CD and 68 healthy control subjects. Twenty two (90%) of the severe CD patients were found to have elevated serum B2-M levels. B2-M levels were significantly higher in all CD patients. However, such a correlation could not be assessed in UC patients. The researchers claimed that B2-M serum levels could prove to be a useful marker in assessing the activity, severity, extent of CD, and treatment efficacy.

To investigate the clinical significance of B2-M in other inflammatory diseases, Kim *et al*[26] searched for B2-M in the serum of 100 SLE patients and found a positive correlation between B2-M serum levels and disease activity. Aygündüz *et al*[27] investigated the relative efficiency of B2-M levels as a marker of disease activity in 43 patients with Behçet's disease. In that study, serum B2-M levels could be regarded as a discriminative marker of activation in Behçet's disease.

Searching Pubmed for relevant studies, we did not find a positive correlation between B2-M serum levels and UC activity. In the present study, we demonstrated that active IBD patients had elevated serum B2-M levels in comparison with inactive patients and the control group. Serum B2-M activity had higher sensitivity, specificity, and predictive values in active IBD patients. Increased B2-M activity in patients with active IBD may support the role of activated macrophages and T-lymphocytes in the disease pathophysiology.

In summary, our study demonstrates that in patients with IBD, serum B2-M level is associated with active disease. B2-M level may be considered a useful marker of IBD and could be a potential indicator of disease activation. B2-M serum level provides additional data to supplement existing markers such as CRP and ESR.

COMMENTS

Background
There are controversies regarding the role of beta 2 microglobulin (B2-M) in inflammatory bowel disease (IBD). In this study we examined B2-M serum levels in patients suffering from the disease to assess its extent, and the possible correlation between serum levels and disease activity.

Research frontier
There is currently increasing interest in research focused on new, more effective, non-invasive biochemical markers for evaluating endoscopic activity in patients with IBD. This study analyzes the association between B2-M levels and disease activity in IBD patients.

Innovations and breakthroughs
This was an original study assessing the relationship between B2-M level and activity of IBD. This question was not described thoroughly enough in the medical literature.

Applications
These findings suggest that B2-M can be used as surrogate marker for activity in IBD patients. B2-M is a simple, inexpensive and objective tool for the assessment of mucosal inflammation.

Terminology

Clinical IBD activity is difficult to assess objectively because of several subjective components. Serum B2-M levels are elevated in diseases associated with increased cell turnover, and they are also elevated in several benign condition such as chronic inflammation.

Peer review

This manuscript contains potentially interesting observations, and should be received for publication.

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RETROSPECTIVE STUDY

Retrieval-balloon-assisted enterography for ERCP after Billroth Ⅱ gastroenterostomy and Braun anastomosis

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Abstract

AIM: To describe an optimal route to the Braun anastomosis including the use of retrieval-balloon-assisted enterography.

METHODS: Patients who received a Billroth Ⅱ gastroenterostomy (*n* = 109) and a Billroth Ⅱ gastroenterostomy with Braun anastomosis (*n* = 20) between January 2009 and May 2013 were analyzed in this study. Endoscopic retrograde cholangiopancreatography (ERCP) was performed under fluoroscopic control using a total length of 120 cm oblique-viewing duodenoscope with a 3.7-mm diameter working channel. For this procedure, we used a triple-lumen retrieval balloon catheter in which a 0.035-inch guidewire could be inserted into the "open-channel" guidewire lumen while the balloon could be simultaneously injected and inflated through the other 2 lumens.

RESULTS: For the patients with Billroth Ⅱ gastroenterostomy and Braun anastomosis, successful access to the papilla was gained in 17 patients (85%) and there was therapeutic success in 16 patients (80%). One patient had afferent loop perforation, but postoperative bleeding did not occur. For Billroth Ⅱ gastroenterostomy, there was failure in accessing the papilla in 15 patients (13.8%). ERCP was unsuccessful because of tumor infiltration (6 patients), a long afferent loop (9 patients), and cannulation failure (4 patients). The papilla was successfully accessed in 94 patients (86.2%), and there was therapeutic success in 90 patients (82.6%). Afferent loop perforation did not occur in any of these patients. One patient had hemorrhage 2 h after ERCP, which was successfully managed with conservative treatment.

CONCLUSION: Retrieval-balloon-assisted enterography along an optimal route may improve the ERCP success rate after Billroth Ⅱ gastroenterostomy and Braun anastomosis.

Key words: Retrieval-balloon-assisted enterography; Billroth Ⅱ gastroenterostomy; Braun anastomosis; Optimal enterography route; Gastrojejunal anastomosis; Efferent loop; Endoscopic retrograde cholangiopancreatography; Duodenoscope; Enterography success rate; Therapeutic success rate

Core tip: For patients with a Billroth Ⅱ gastroenterostomy, endoscopic retrograde cholangiopancreatography (ERCP) is difficult because of altered anatomy, and the success rate decreases for those with Braun anastomosis. ERCP failure in such patients is caused by difficulties in entering the afferent loop and accessing the papilla. We reported the use of a wire-guided retrieval balloon to remove common bile duct stones and facilitate endoscope insertion for successful ERCP in post-gastrointestinal surgery patients. We termed the procedure "retrieval-balloon-assisted enterography". We believe that retrieval-balloon-assisted enterography along the optimal route may improve the ERCP success rate after Billroth Ⅱ gastroenterostomy and Braun anastomosis.

INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is very useful in pancreaticobiliary disease management in patients with a normal anatomy and surgically altered anatomy. For patients with Billroth Ⅱ gastroenterostomy, ERCP is difficult because of the altered anatomy, and the success rate decreases for those with Braun anastomosis. The success rate was reported to be 83% for simple Billroth Ⅱ gastroenterostomy but only 29% in patients with a Billroth Ⅱ gastroenterostomy and an additional Braun anastomosis[1]. ERCP failure in such patients was caused by difficulties in entering the afferent loop and accessing the papilla[2-4]. Recently, we reported the use of a wire-guided retrieval balloon to remove common bile duct stones and facilitate endoscope insertion for successful ERCP in post-gastrointestinal surgery patients[5,6]. We termed the procedure "retrieval-balloon-assisted enterography". In this retrospective study, we aimed to describe endoscope insertion technique for patients with a Billroth Ⅱ gastroenterostomy and Braun anastomosis.

An institutional review board-approved retrospective analysis was performed at the Department of General Surgery, Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University. In this study, we included patients who underwent Billroth Ⅱ gastroenterostomy and Braun anastomosis for therapeutic biliary interventions between January 2009 and May 2013 in the ERCP unit in the Department of General Surgery. During this period, 2994 ERCP procedures were performed. Patients who had normal anatomy and other surgically altered anatomy were excluded from the study. The study included 109 patients who had a Billroth Ⅱ gastroenterostomy and 20 patients who had a Billroth Ⅱ gastroenterostomy with Braun anastomosis.
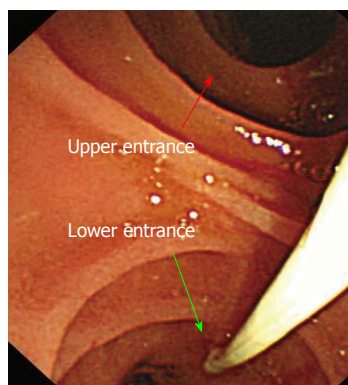
Figure 1 Gastrojejunal anastomosis is detected at the distal end of the stomach, and 2 stomal openings corresponding to an end-to-side anastomosis can be identified endoscopically. If the efferent loop was constructed at the greater curvature of the stomach in the previous surgery, the "lower entrance" is the entrance to the right efferent loop.

MATERIALS AND METHODS

Endoscopic procedures

The procedure was performed with the patient under pharyngeal anesthesia, and sedation with an intramuscular injection of 10 mg diazepam. All the patients received oxygen and were monitored by electrocardiography and pulse oximetry. The patient was placed in the prone position. ERCP was performed under fluoroscopic control using a total length of 120 cm oblique-viewing duodenoscope with a diameter of 3.7 mm working channel (Olympus V260, Olympus Medical Systems, Tokyo, Japan). For this procedure, we used a triple-lumen retrieval balloon catheter (Extractor Pro RX retrieval balloon catheter, Boston Scientific, Shanghai, China) in which a 0.035-inch (0.089 mm) guidewire could be inserted into the "open-channel" guidewire lumen while the balloon could be simultaneously injected and inflated through the other 2 lumens.

In our technique, we first review the patient's previous surgical records, which most often indicate that the efferent loop is at the greater curvature of the stomach. The solution is to extend the duodenoscope along the greater stomach curvature until the gastrojejunal anastomosis becomes visible, from which perspective the "lower entrance" is the correct efferent loop (Figure 1). The efferent loop is a better entrance for the duodenoscope because it is less angulated than the afferent loop. In such cases, our solution is to extend the duodenoscope along the efferent loop until the Braun anastomosis becomes visible. Three stomal openings can be visualized endoscopically, but identifying the correct entrance is a major challenge (Figure 2). The "middle entrance" is the entrance to the loop that can be used to reach the papilla of Vater when the endoscope is advanced from the efferent loop and is unique irrespective of the endoscopic approach used. The endoscope tip appears to be located in the difficult-to-negotiate portion when all 3 stomal openings are visible at the Braun anastomosis site; thereafter, we insert the guidewire of the retrieval balloon catheter
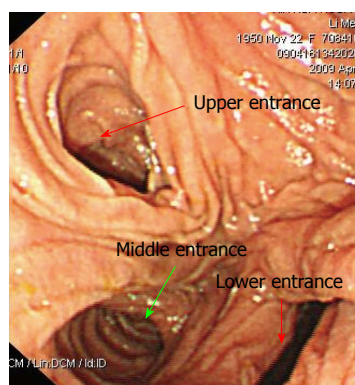
Figure 2 Three stomal openings can be identified endoscopically at the site of the Braun anastomosis, and the "middle entrance" leads to the appropriate loop to reach the papilla of Vater. The "middle entrance" is unique irrespective of the endoscopic approach used.

Figure 4 Retrieval-balloon-assisted enterography. A catheter is advanced into the middle limb and contrast injected into the loop to confirm that the limb is the duodenal stump.

Figure 3 We inserted the guidewire of retrieval balloon catheter to the "middle entrance", and the retrieval balloon catheter is inserted over the guidewire.
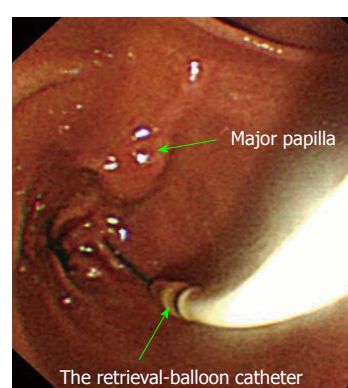
Figure 5 Endoscope is then propelled slightly forward, while at the same time the retrieval-balloon catheter is strongly retracted into the working channel to allow the scope to advance. Then, the endoscope is straightened and advanced to the major papilla.

through the "middle entrance" and then insert the retrieval balloon catheter over the guidewire (Figure 3). The balloon is hooked to the duodenal limb which is inflated. The retrieval balloon catheter can be used to explore the limb tract by injecting a contrast medium through the catheter under radiography and then confirming whether the limb is a duodenal stump (Figure 4). By laying it on the duodenal stump as a guide, the retrieval-balloon catheter could also be used to prevent the duodenoscope from sliding out of the right limb into another limb upon forward motion. After successful access to the right limb is achieved, the retrieval balloon is visible within the tract ahead, instead of emerging from it. While the retrieval-balloon catheter is strongly retracted into the working channel to allow the scope to advance, the endoscope is then propelled slightly forward to the major papilla (Figure 5).

RESULTS

The study group included 109 patients who had a simple Billroth Ⅱ gastroenterostomy (102 male, 21 female; mean age, 69.4 years; age range, 47-90 years) and 20 patients

who underwent Billroth Ⅱ gastroenterostomy and Braun anastomosis (16 male, 4 female; mean age, 72.4 years; age range, 55-86 years). Table 1 summarizes the demographic and clinical characteristics of the patients and the interventions they received. ERCP was unsuccessful in 3 patients because of failure to access the papilla due to the presence of a long afferent loop, and the procedure was unsuccessful in 1 patient because of cannulation failure as the papilla of Vater could not be located in the duodenal stump. The papilla was successfully reached in 17 patients (85%), and there was therapeutic success in 16 patients (80%). One patient had afferent loop perforation, underwent laparotomy, and was discharged 2 wk later. Postoperative bleeding did not occur.

For Billroth Ⅱ gastroenterostomy, there was failure in accessing the papilla in 15 patients (13.8%). ERCP was unsuccessful because of tumor infiltration (6 patients), a long afferent loop (9 patients), and cannulation failure (4 patients). The papilla was successfully accessed in 94 patients (86.2%), and there was therapeutic success in 90 patients (82.6%). Afferent loop perforation did not occur in any of these patients. One patient had hemorrhage 2 h after ERCP, which was successfully managed with con-

Table 1 Demographic and clinical characteristics of the patients 20 patients who had a Billroth Ⅱ gastroenterostomy and Braun anastomosis

Cases	Sex	Age (yr)	Previous surgery	Indication	Enterography success	Cannulation success	Intervention
1	M	61	Gastric cancer	Cholangitis, CBD stone	Yes	Yes	Stone extraction
2	M	83	Peptic ulcer	Cholangitis, CBD stone	Yes	No	/
3	M	77	Gastric cancer	Jaundice, cholestasis	Yes	Yes	Stone extraction
4	M	86	Peptic ulcer	Cholangitis, CBD stone	Yes	Yes	Drainage (stent)
5	M	72	Gastric cancer	Cholangitis, CBD stone	Yes	Yes	Stone extraction
6	M	63	Gastric cancer + CBD exploration	Cholangitis, CBD stone	No	/	/
7	F	59	Gastric cancer	Cholangitis, CBD stone	Yes	Yes	Drainage (stent)
8	M	77	Gastric cancer	Jaundice, cholestasis	No	/	/
9	M	85	Gastric cancer	Jaundice, cholestasis	Yes	Yes	Drainage (stent)
10	F	58	Gastric cancer	Cholangitis, CBD stone	Yes	Yes	Drainage (NBD)
11	F	66	Gastric cancer	Jaundice, cholestasis	No	/	/
12	M	84	Gastric cancer	Jaundice, cholestasis	Yes	Yes	Drainage (stent)
13	M	84	Gastric cancer	Jaundice, cholestasis	Yes	Yes	Drainage (NBD)
14	M	55	Gastric cancer	Cholangitis, CBD stone	Yes	Yes	Stone extraction
15	F	69	Gastric cancer	Cholangitis, CBD stone	Yes	Yes	Stone extraction
16	M	72	Gastric cancer	Cholangitis, CBD stone	Yes	Yes	Drainage (stent)
17	M	80	Peptic ulcer	Cholangitis, CBD stone	Yes	Yes	Stone extraction
18	M	58	Gastric cancer	Jaundice, cholestasis	Yes	Yes	Drainage (stent)
19	M	83	Gastric cancer	Jaundice, cholestasis	Yes	Yes	Drainage (stent)
20	M	76	Gastric cancer	Jaundice, cholestasis	Yes	Yes	Drainage (stent)

CBD: Common bile duct.

servative treatment.

DISCUSSION

Retrieval-balloon-assisted enterography improves the overall success rate in reaching the papilla in ERCP after Billroth Ⅱ gastroenterostomy and Braun anastomosis. The previously reported success rate was approximately 30% in patients who had a Billroth Ⅱ gastroenterostomy with Braun anastomosis[1,7]. ERCP failure in these patients was due to failure in entering the afferent loop and the increased number of anastomoses. Thus far, no reports have indicated the optimal enterography route in which cannulation can be performed. For patients with Billroth Ⅱ gastroenterostomy and Braun anastomosis, we describe our experience in establishing an ERCP enterography route. We first review the patient's previous surgical records, which most often indicate that the efferent loop is at the greater curvature of the stomach. One major challenge is distinguishing between the afferent and efferent loops. The solution is to extend the duodenoscope along the greater curvature of the stomach until the gastrojejunal anastomosis becomes visible, from which perspective the "lower entrance" in endoscopic image is the correct efferent loop. Occasionally, we are able to draw back the duodenoscope along the greater curvature of the stomach to "relax" the gastrojejunal anastomosis and thus differentiate the "upper entrance" from the "lower entrance." The efferent loop makes a better entrance for the duodenoscope because it is less angulated than the afferent loop. Thus, this route is useful for increasing the success rate of the enterography procedure. Many endoscopists attempt to enter the afferent loop *via* the site of the gastrojejunal anastomosis, but the sharp angulation caused by adhesions may make it impossible to advance the endoscope to the afferent loop. Premature entry into the afferent loop at the gastrojejunal anastomosis is the main cause of failure to access the papilla. Three stomal openings can be visualized endoscopically at the site of the Braun anastomosis, but identifying the correct entrance is a major challenge. The "middle entrance" is the entrance to the loop that can be used to reach the papilla of Vater when the endoscope is advanced from the efferent loop and is invariable irrespective of the endoscopic approach used. We recommend extending the duodenoscope along the greater curvature of the stomach until the gastrojejunal anastomosis, then advancing the endoscope through the "lower entrance", along this efferent loop to the Braun anastomosis, and the "middle entrance" is the correct entrance to reach the papilla of Vater. For patients with Billroth Ⅱ gastroenterostomy and Braun anastomosis, We believe that this is the optimal ERCP enterography route (Figure 6)[8].

As in patients with normal anatomy[9,10], anterior oblique-viewing endoscopes[11,12], side-viewing endoscopes[3,13-16], forward-viewing gastroscopes[17], and multibending endoscopes[18] have been reported in previous studies on ERCP for those patients with prior Billroth Ⅱ gastrectomy. It was reported that the success rate of reaching the papilla of Vater ranges from 63% to 92%[1,2,4,15,19]. In our study, the overall success rate in reaching the papilla was 86.2%, and 82.6% patients had therapeutic success using a conventional duodenoscope for ERCP, which is a little better than and quite comparable with the success rates in previous reports. In patients who have had a Braun anastomosis, the ERCP failure rate is reported to be 70%[1]. A long afferent loop and the Braun anastomosis are the main reasons of failure in accessing the papilla. To
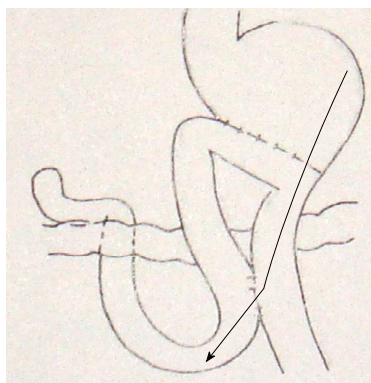
Figure 6 Optimal enterography routes for endoscopic retrograde cholangiopancreatography in patients with a Billroth II gastroenterostomy and a Braun anastomosis.

solve these altered anatomy challenges, a stiffer endoscope along with manual compression has been used to access the papilla. It has been suggested to increase the success rate in accessing the papilla and avoiding loop formation at the ligament of Treitz[1]. Insufficient stiffness of the endoscope may be an important cause of failure in accessing the papilla. Instruments such as a polypectomy snare can be placed into the endoscope to increase the overall stiffness during intubation. Alternative methods such as cap-assisted or double-balloon endoscopy can also improve the ERCP success rate in those patients with Billroth II gastrectomy and Braun anastomosis[20,21]. In our technique, the guidewire of the retrieval balloon is advanced to the right limb. Subsequently, a retrieval balloon is inserted over the guidewire. We use this retrieval balloon to explore the right limb by using contrast enhancement to observe the tract of the limb on the radiographic images. The balloon is hooked to the duodenal limb which is inflated, and not only indicates the direction of the tract to guide the endoscope forward but also facilitates the forward movement of the endoscope with fewer injuries to the intestinal wall. While the balloon catheter is strongly retracted into the working channel to allow the scope to advance, the endoscope is then propelled slightly forward. By laying it on the right limb, the retrieval balloon catheter could also be used as a guide to prevent the duodenoscope from sliding out of the right limb into another limb upon forward motion. After successful access of the right limb is achieved, the retrieval balloon becomes visible within the tract ahead, instead of emerging from it. This is particularly important at the Braun anastomosis site, where 3 stomal openings can be endoscopically identified; thus, retrieval-balloon-assisted enterography could ensure the success of ERCP. For patients with a Billroth II gastroenterostomy and Braun anastomosis, the overall success rate in reaching the papilla was 85%, and the therapeutic success rate was 80% in this study.

We recommend extending the duodenoscope along the greater curvature of the stomach to the gastrojejunal anastomosis, then advancing the endoscope through the "lower entrance", along this efferent loop to the Braun anastomosis, and the "middle entrance" is the correct entrance

to reach the papilla of Vater. For patients with Billroth II gastroenterostomy and Braun anastomosis, we believe that this is the optimal ERCP enterography route. Retrieval-balloon-assisted enterography along the optimal route may improve the ERCP success rate in these patients. This study was retrospective, reflecting the experience of a single center and suggesting that the reproducibility of this technique should be assessed in prospective studies in the future.

COMMENTS

Background
Endoscopic retrograde cholangiopancreatography (ERCP) is very practical in pancreaticobiliary disease management in patients with normal anatomy and surgically altered anatomy. The success rate was reported to be 83% in patients with a Billroth II gastroenterostomy but only 29% with a Billroth II gastroenterostomy and an additional Braun anastomosis. ERCP failure in such patients was caused by difficulties in entering the afferent loop and accessing the papilla. In this retrospective study, we described the use of retrieval-balloon-assisted enterography along an optimal route in challenging patients with a Billroth II gastroenterostomy and Braun anastomosis.

Research frontiers
Anterior oblique-viewing endoscopes, side-viewing endoscopes, forward-viewing gastroscopes, and multibinding endoscopes have been used in most studies on ERCP for patients with prior Billroth II gastrectomy. The success rate of reaching the papilla is 63%-92%. In patients who have had a Braun anastomosis, ERCP failure rate is reported to be 70%.

Innovations and breakthroughs
The authors reported the use of a wire-guided retrieval balloon to remove common bile duct stones and facilitate endoscope insertion for successful ERCP in post-gastrointestinal surgery patients. They termed the procedure retrieval-balloon-assisted enterography. They believe that retrieval-balloon-assisted enterography along an optimal route may improve the ERCP success rate in patients with a Billroth II gastroenterostomy and Braun anastomosis.

Applications
Retrieval-balloon-assisted enterography along the optimal route may improve the ERCP success rate in patients with a Billroth II gastroenterostomy and Braun anastomosis.

Terminology
Braun anastomosis is an anastomosis between the afferent and efferent loops of the jejunum after a loop gastroenterostomy.

Peer review
This manuscript is a retrospective study and the sequel of a prior publication. The technique is very interesting and the results are very encouraging but it reflects only a single center experience. For this reason the results are not valid but it would be interesting to start a prospective study on this topic to valid the procedure. However, the study suggests a new therapeutic possibility to perform ERCP in patients with Billroth II that can be reproduced in other Centers and for this reason it could be published.

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RETROSPECTIVE STUDY

Risk factors associated with missed colorectal flat adenoma: A multicenter retrospective tandem colonoscopy study

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Abstract

AIM: To determine the miss rate for colorectal flat adenomas during colonoscopy and the risk factors.

METHODS: Flat adenomas are frequently missed during colonoscopy. However, the risk factors that influence their miss rates are unclear. This was a multicenter, retrospective study in which patients diagnosed with colorectal adenomas at a diagnostic colonoscopy and followed within 3 mo by a second therapeutic colonoscopy were pooled out from the established database. The "per-patient" and "per-adenoma" adenoma miss rates (AMR) for overall adenomas and flat adenomas, and patient-, adenoma-, and procedure-related risk factors potentially associated with the "per-adenoma" AMR for flat adenomas were determined.

RESULTS: Chromoscopy and high-definition colonoscopy were not taken under consideration in the study. Among 2093 patients with colorectal adenomas, 691 (33.0%) were diagnosed with flat adenomas, 514 with concomitant protruding adenomas and 177 without. The "per-patient" AMR for flat adenomas was 43.3% (299/691); the rates were 54.3% and 11.3%, respectively, for those with protruding adenomas and those without (OR = 9.320, 95%CI: 5.672-15.314, χ^2 = 99.084, $P < 0.001$). The "per-adenoma" AMR for flat adenomas was 44.3% (406/916). In multivariate analysis, older age, presence of concomitant protruding adenomas, poor bowel preparation, smaller adenoma size, location at the right colon, insufficient experience of the colonoscopist, and withdrawal time < 6 min were associated with an increased "per-adenoma" AMR for flat adenomas. The AMR for flat adenomas was moderately correlated with that for overall adenomas (r = 0.516, P < 0.0001). The AMR for flat adenomas during colonoscopy was high.

CONCLUSION: Patient's age, concomitant protruding adenomas, bowel preparation, size and location of adenomas, proficiency of the colonoscopist, and withdrawal time are factors affecting the "per-adenoma" AMR for flat adenomas.

Key words: Flat adenoma; Colorectal cancer; Miss rate; Risk factor; Colonoscopy

Core tip: The miss rate for flat adenomas during colo-

noscopy is high. Patient's age, concomitant protruding adenomas, bowel preparation, size and location of adenomas, proficiency of the colonoscopist, and withdrawal time are factors affecting the "per-adenoma" adenoma miss rate for flat adenomas.

INTRODUCTION

Colorectal cancer (CRC) is the most common malignant tumor and the second leading cause of cancer-related deaths in the world[1]. CRC mainly originates from colorectal adenomas[2]. According to the morphology, Kudo *et al*[3] classified colorectal adenomas into protruding and flat ones. It has been reported that colorectal flat adenomas have a greater tendency to develop into severe dysplasia and carcinoma than protruding adenomas[4,5]. However, because of their flat morphology and low awareness among colonoscopists, many flat adenomas are missed during colonoscopy although they are often visible. Therefore, flat adenomas are not only difficult to detect, but also easy to miss[6-8]. Specifically, the miss rates for flat adenomas during colonoscopy range from 35% to 60%, which are much higher than those (4%-19%) seen in the protruding type of adenomas[6,9,10]. It is believed that undetected or missed adenomas may play an important role in the incidence of interval cancers[11].

Colonoscopy has been considered as the "golden standard" in detection of colorectal adenomas and plays an important role in CRC prevention. In addition, colonoscopic polypectomy with follow-up monitoring has been proven to decrease the incidence of colorectal cancer, mainly in the left colon[12]. However, colorectal adenomas, especially flat adenomas, are frequently missed during colonoscopy as mentioned above. Flat adenomas are frequently localized at the right part of the colon, and it has been suggested that the high miss rate for flat adenomas at the right colon contributes to the high incidence of cancer at the right colon after colonoscopy[13]. Therefore, it is extremely important and essential to recognize and identify these flat neoplastic lesions at an early stage. The use of new techniques such as chromoendoscopy or magnifying narrow-band imaging during colonoscopy in recent years appears to significantly improve the detection of colorectal flat adenomas during colonoscopy[4,10,13,14]; however, controversy exists[9,15].

It is critical to determine the miss rate of colonoscopy for colorectal flat adenomas, and more importantly, to identify the risk factors that are associated with the in-creased flat adenoma miss rates. However, there are only few studies evaluating the miss rates of flat adenomas[9,15]. Moreover, the risk factors influencing the miss rate for flat adenomas have not been explored and thus are not understood. Therefore, this multicenter study aimed to determine the miss rates in detection of colorectal flat adenomas during colonoscopy and the risk factors that influence the miss rates.

MATERIALS AND METHODS

Sources of patients

This was a multicenter, retrospective study in which patients diagnosed with colorectal adenomas at a diagnostic colonoscopy and followed within 3 mo by a second therapeutic colonoscopy[16] between September 2009 and September 2011 from four Chinese hospitals were pooled out from the database established in the computerized system for colonoscopy. The study proposal was approved by the ethics committees of these four institutions (Medical Ethics Committee of Nanfang Hospital, Southern Medical University; Medical Ethics Committee of Wuxi City People's Hospital Affiliated with Nanjing Medical University; Ethics Committee of Mianyang Central Hospital; Medical Ethics Committee of Shenzhen Longgang Central Hospital). All patients gave written informed consent at the first and second colonoscopy to allow their colonoscopy data to be used for this research purpose.

Selection criteria for patients

The patients enrolled in this study had to meet the following criteria: (1) they were 18 years old or over; (2) the colonoscope reached the cecum (*i.e.*, completion of colonoscopy); (3) the interval duration between the first and second colonoscopy procedures was less than 90 d; the first colonoscopy was only for diagnosis, and the second colonoscopy was for therapeutic purpose and with good bowel preparation; (4) colonic images were properly taken at various parts of the colon; it was essential that the cecum, appendiceal orifice or ileocecal valve was clearly pictured after insertion of the colonoscope (to indicate the completion of the colonoscope insertion) and the images of the rectum were properly taken during the withdrawal of the colonoscope (to ensure calculation of the withdrawal time); and (5) the colonoscopists at the first colonoscopy had performed normal total colonoscopy on more than 100 cases; the colonoscopists at the second colonoscopy had performed colonoscopy on more than 1000 cases, with more than 150 cases annually. Cases with colorectal cancer, polyposis syndrome (defined as conditions where a patient had more than 100 polyps in the intestine, including familial polyposis syndrome, serrated polyposis syndrome, Peutz-Jeghers syndrome, juvenile polyposis syndrome, and Cronkhite-Canada syndrome), inflammatory bowel disease, partial large bowel resection or insufficient data required for analysis were excluded from this study.

Colonoscopy and imaging procedures

Large bowel preparation was performed by using polyethylene glycol electrolyte solution, sodium phosphate or mannitol based on the hospital practice and guidelines. Patients were examined first in the left lateral decubitus position. Then, the patient was placed in the supine position or on the right side as needed to facilitate intubation of the cecum. Colonoscopy procedures were carried out by using electronic colonoscopies CF-240 I and CF-260 I (Olympus, Tokyo, Japan) in our study. In cases with areas suspicious of adenoma, dye solution (3-6 mL of 0.2% indigo carmine, Nanjing Weichuang Medical Technology Co., Ltd., Nanjing, China) was sprayed directly onto the areas with a 20 mL syringe to allow a cushion of air to push the dye through the biopsy channel. Images of any lesions were taken. Endoscopic mucosal resection, endoscopic piecemeal mucosal resection or endoscopic submucosal dissection was used for endoscopic resection of flat adenomas.

Study endpoints

The primary endpoints were the miss rate and the associated risk factors of flat adenoma. The second colonoscopy was used as the reference standard, and any adenomas that were identified at the first colonoscopy were not counted at the second colonoscopy. Thus, all adenomas that were identified at the second colonoscopy but not detected at the first colonoscopy were defined as missed adenomas. The adenoma miss rate (AMR) was calculated using both patient and adenoma based analyses. The "per-patient" AMR was calculated by the number of patients with missed adenoma(s) divided by the total number of patients examined. The "per-adenoma" AMR was calculated by the number of missed adenoma(s) divided by the total number of adenomas detected at both examinations. Accordingly, the AMR for flat adenomas was also calculated.

The risk factors potentially associated with the miss of adenomas, flat adenomas in particular, included in the study were patient-related, such as demographic and clinical characteristics, and quality of bowel preparation; adenoma-related, such as the size and location, and pathologic classification; and procedure-related, such as proficiency and specialty of the colonoscopist, colonoscopy operative mode, and withdrawal time. A total of 29 colonoscopists were involved in this study. The proficiency of colonoscopists was defined by the cumulative cases of colonoscopy as (1) more than 1000; (2) between 500 to 1000; and (3) less than 500 cases. The specialty of colonoscopists was defined as gastroenterology *and non-g*astroenterology. Quality of bowel preparation was assessed by the colonoscopist at colonoscopy, which was graded as being excellent (no or minimal solid stool and only clear fluid requiring suction), adequate (collections of semi-solid debris that are cleared with washing/suction) or poor (solid or semi-solid debris that cannot be cleared) as previously described[17]. Cases with excellent and adequate bowel preparation were grouped together as good bowel preparation.

The mode of colonoscopy operation was defined as one-person (*i.e.*, only the endoscopist personally advanced the endoscope during insertion.) or two-person (*i.e.*, colonoscopy was performed with a nurse or assistant actively advancing the colonoscope during insertion) technique. The withdrawal time was defined as the time taken for colonoscopy withdrawal to the rectum minus the time taken for colonoscopy insertion to the cecum based on the times recorded on the images. The withdrawal time of the colonoscopy for a particular colonoscopist was represented by the average time of at least 100 normal total diagnostic colonoscopy procedures (*i.e.*, no lesions were detected) performed by that colonoscopist as recorded in the database as previously described[18].

The adenoma size was measured by the colonoscopist during the colonoscopy using the opening aperture of a biopsy forceps (6 mm as a cut-off value), or after resection and recorded in the colonoscopy reports. The locations of adenomas were defined as the right colon (including the cecum, ascending colon, transverse colon, and hepatic flexure), the left colon (including the sigmoid colon, descending colon and splenic flexure) and the rectum. Adenoma location was estimated using anatomic landmarks and insertion distances. The Japanese Research For Cancer of Colon and Rectum Classification was applied to classify lesions as protruding and flat including flat elevated and flat depressed) lesions[3]. Flat elevated lesions were defined as those with a height less than half of the lesion in diameter[19]. Flat depressed lesions were defined as those with a central distinct depression[20]. Two experienced endoscopists verified the morphology from the photo documentation for a representative group of adenomas that were randomly selected from the analyzed samples. Pathological classifications were interpreted by pathologists using the WHO and Vienna criteria for colorectal adenoma[21]. The serrated adenomas including sessile serrated adenomas/polyps and traditional serrated adenomas were diagnosed as recommended by the WHO[22], and intraepithelial dysplasia was defined as low and high grade, depending on the glandular complexity, extent of nuclear stratification, and severity of nuclear morphology[23]. Advanced adenomas were defined as tubular adenomas of at least 10 mm in diameter (including serrated adenomas) or as adenomas containing villous or tubulovillous histological characteristics, high grade dysplasia, or any combination thereof[24].

Statistical analysis

Continuous variables are expressed as mean ± SD, and categorical variables are expressed as proportion or percentage. Comparisons among multiple groups with continuous variables were performed by analysis of variance. The χ^2 test was used to determine the association between potential risk factors and miss of adenoma. The Bonferroni test was used for multiple comparisons. The multivariate logistic regression analysis was used to determine the independent risk factors for flat adenoma.

4567 patients underwent two consecutive colonoscopies

Colonoscopic images did not meet the requirements or provided insufficient information (1473)

Colonoscopist or bowel preparation did not meet the requirements (378)

Interval time > 90 d (142)

Colorectal cancer (44)

With previous colon resection (163)

Colonoscope did not reach the ileocecal valve (21)

Inflammatory bowel disease (186)

Colon polyposis syndrome (67)

2093 cases with colorectal adenomas, including 691 with flat adenoma underwent the second colonoscopy within the next 90 d

Figure 1 Flowchart of patient screening in this study. A total of 2093 patients with adenomas satisfied the inclusion and exclusion criteria, including 691 with flat adenomas (514 with and 177 without protruding adenomas), and 1402 with only protruding adenomas.

To establish a group of missed adenomas and a group of diagnosed adenomas, one of the adenomas missed at the first colonoscopy was selected from each of patients with missed adenoma(s) and one of the adenomas detected at the first colonoscopy selected from each of patients without any missed adenoma, by using a simple random sampling method. Then, a multivariate analysis model was developed to determine independent risk factors associated with an increased AMR for colorectal flat adenoma. The Spearman correlation analysis was used to analyze the correlation between the "per-adenoma" AMR for overall adenomas and that for flat adenomas, as previously used for determination of the correlation between the detection rate for overall adenomas and that for flat adenomas by Reinhart *et al*[25]. A *P* value < 0.05 was considered statistically significant. SPSS 17.0 (IBM, United States) statistical software was employed in this study.

RESULTS

Demographic and clinical characteristics of patients with flat adenomas and protruding adenomas

Overall, 4567 patients who underwent two consecutive colonoscopies were screened from the data system, of which 2474 were ineligible for the study, and thus 2093 patients with colorectal adenomas were included in the study (Figure 1). Among these 2093 patients, 691 (33.0%) were diagnosed with flat adenoma(s), including 177 without protruding adenoma and 514 with protruding adenoma. The remaining 1402 (67%) cases were diagnosed with

protruding adenomas without flat adenomas. Among the 177 patients only with flat adenomas and those with flat adenomas and concomitant protruding adenomas, males accounted for 51.4% and 64%, respectively. Among the 514 patients with flat adenomas and concomitant protruding adenomas, 222 (43.2%) had more than three adenomas at the first colonoscopy, which was significantly greater than that in other groups (Table 1).

Clinical and pathologic features of flat adenomas vs protruding adenomas

In total, 4632 adenomas were detected, including 3665 detected at the first colonoscopy and 967 detected at the second colonoscopy but missed at the first colonoscopy. There were 916 flat adenomas (19.8%) and 3716 protruding adenomas (80.2%). Compared with the protruding colorectal adenomas, the flat adenomas were mainly localized in the right colon (410/916; 44.8%), and smaller in size [most (744/916, 81.2%) were less than 10 mm] (Table 2). Pathologically, the majority of flat adenomas (745/916; 81.3%) were tubular adenomas, followed by tubulovillous or villous adenoma (151/916; 16.3%) and serrated adenomas (20/916; 2.2%). The proportion of a villous structure in patients with flat adenomas was more than that in protruding adenoma (16.5% *vs* 13.9%, *P* = 0.041). Compared with the protruding adenomas, the flat adenomas were more associated with high grade dysplasia adenoma (7.5% *vs* 5.2%, *P* = 0.006) (Table 2). Among the 916 flat adenomas, 906 (98.9%) were classified as flat elevated and 10 (1.1%) as flat depressed adenomas. Flat elevated and flat depressed adenomas were mainly located at the right colon (44.7% *vs* 50.0%). However, more flat depressed adenomas had an adenoma size of 6-9 mm (70.0% *vs* 42.4%), a pathologically tubulovillous and villous type (50.0% *vs* 16.1%) and a high-grade dysplasia (40.0% *vs* 7.2%), compared with flat elevated adenomas. However, due to the small number of cases with flat depressed adenomas, no further analysis was conducted to compare the two types of flat adenomas.

Miss rates for colorectal adenomas during colonoscopy

Among the 2093 patients, "missed" adenomas were observed in 560, and thus the overall "per-patient" AMR was 26.8%. Accordingly, the "per-patient" AMR were 43.3% (299/691) in patients with flat adenomas; the rates were 54.3% (279/514) and 11.3% (20/177), respectively, for those with concomitant protruding adenomas and those without (OR = 9.320, 95%CI: 5.672-15.314, χ^2 = 99.084, *P* < 0.001). The "per-patient" AMR was 18.6% (261/1402) for those with only protruding adenomas, which was significantly lower than that for those with flat adenomas (OR = 0.300, 95%CI: 0.245-0.367, χ^2 = 143.566, *P* < 0.001).

Among the 4632 adenomas, 967 were missed at the first colonoscopy, and thus the overall "per-adenoma" AMR was 20.9%. Accordingly, the "per-adenoma" AMRs were 44.3% (406/916) and 15.1% (561/3716), respectively, for flat and protruding adenomas (OR = 4.477,

Table 1 Demographic and clinical characteristics of patients with different types of colorectal adenomas *n* (%)

Patients	Total (*n* = 2093)	Flat adenoma (*n* = 177)	Flat and protruding adenoma (*n* = 514)	Protruding adenoma (*n* = 1402)	*P* value
Sex					
Male	1369	91 (51.4)	329 (64.0)	949 (67.7)	< 0.001
Female	724	86 (48.6)	185 (34.0)	453 (32.3)	< 0.001
Age (mean ± SD, yr)		55.91 ± 12.68	59.30 ± 12.47	54.78 ± 13.24	< 0.001
Alarm symptoms	1621	134 (75.7)	388 (75.5)	1099 (78.4)	0.341
Diverticulosis	123	10 (5.6)	27 (5.3)	86 (6.1)	0.761
Family history of CRC	131	11 (6.2)	32 (6.2)	88 (6.3)	0.999
History of adenomas	475	40 (22.6)	139 (27.0)	296 (21.1)	0.023
History of abdominal surgery	149	3 (1.7)	35 (6.8)	111 (7.9)	0.010
Cases with adenomas ≥ 3 at first colonoscopy	458	8 (4.5)	222 (43.2)	228 (16.3)	< 0.001

Family history of colorectal cancer (CRC) was defined as the development of CRC in one first-degree relative < 50 years or at least two first-degree relatives 50-70 years; Diverticulosis is defined as the presence of ≥ 2 diverticula. Data are expressed as number (%) unless otherwise indicated.

Table 2 Clinicopathologic characteristics of different types of colorectal adenomas *n* (%)

Characteristic	Total (*n* = 4632)	Flat (*n* = 916)	Protruding (*n* = 3716)	χ^2 test	*P* value
Location				61.902	< 0.001
Rectum	1003	184 (20.1)	819 (22.0)		
Left colon	2054	322 (35.2)	1732 (46.6)		
Right colon	1575	410 (44.8)	1165 (31.4)		
Size				95.954	< 0.001
< 6 mm	1223	353 (38.5)	870 (23.4)		
6-9 mm	2159	391 (42.7)	1768 (47.6)		
≥ 10 mm	1250	172 (18.8)	1078 (29.0)		
Pathological classification				13.775	0.001
Tubular	3910	745 (81.3)	3165 (85.2)		
Tubulovillous and villous	666	151 (16.5)	515 (13.9)		
Serrated	56	20 (2.2)	36 (1.0)		
Dysplasia				7.534	0.006
Low	4370	847 (92.5)	3523 (94.8)		
High	262	69 (7.5)	193 (5.2)		
Advanced adenoma				19.606	< 0.001
No	3322	711 (77.6)	2611 (70.3)		
Yes	1310	205 (22.4)	1105 (29.7)		

95%CI: 3.822-5.245, χ^2 = 380.002, *P* < 0.001).

In addition, a total of 205 advanced flat adenomas were diagnosed in 200 patients at the first and second examinations; 24 were missed in 22 patients. Thus, the "per-patient" and "per-adenoma" AMRs for advanced flat adenomas were 11.0% and 11.7%, respectively.

Risk factors associated with the "per-adenoma" AMR for flat adenomas

Associations of potential risk factors related to patients, adenomas, and procedures are summarized in Table 3.

In univariate analysis, older age, presence of concomitant protruding adenomas, poor bowel preparation, smaller size of adenoma, location at the right colon, tubular type, non-advanced adenoma, insufficient experience of the colonoscopist, double operative mode and withdrawal time < 6 min were associated with an increased "per-adenoma" AMR for flat adenomas (Table 3). In the multivariate analysis, all above factors, except for pathological type of adenomas, status of advanced adenoma, non-gastroenterology specialty and double

operative mode, were identified to be independently associated with an increased "per-adenoma" AMR for flat adenomas (Table 3).

Risk factors associated with the "per-adenoma" AMR for advanced flat adenomas

In univariate analysis, an adenoma size less than 10 mm, the location at the right colon, poor bowel preparation, and non-gastroenterology specialty were significantly associated with an increased AMR for advanced flat adenomas. Due to the small number of advanced flat adenomas, multivariate analysis was not performed (Table 4).

Correlation between "per-adenoma" AMR for all adenomas and "per-adenoma" AMR for flat adenomas

The median "per-adenoma" AMRs for overall and flat adenomas obtained by different colonoscopists were 22.3% (interquartile range, 18.37%-26.35%) and 45.65% (interquartile range, 34.48%-60.83%). There was a moderate correlation between the total miss rates for overall adenomas and the miss rates for flat adenomas. The cor-

Table 3 "Per-adenoma" miss rates and the risk factors in detection of flat adenomas during colonoscopy *n* (%)

Risk factor	Total (*n* = 916)	Diagnosed (*n* = 510)	Missed (*n* = 406)	Univariate analysis		Multivariate analysis	
				χ^2 test	*P* value	OR (95%CI)	*P* value
Patient-related							
Age				9.021	0.003		
< 60 (yr)	493	297 (60.2)	196 (39.8)			1	
≥ 60 (yr)	423	213 (50.4)	210 (49.6)			2.062 (1.390-3.061)	< 0.001
Sex				1.586	0.208		
Male	559	302 (54.0)	257 (46.0)				
Female	357	208 (58.3)	149 (41.7)				
Anesthesia				0.197	0.657		
Yes	239	136 (56.9)	103 (43.1)				
No	677	374 (55.2)	303 (44.8)				
History of adenomas							
Yes	243	129 (53.1)	114 (46.9)	0.899	0.343		
No	673	381 (56.6)	292 (43.4)				
Previous surgery				0.105	0.746		
Yes	45	24 (53.3)	21 (46.7)				
No	871	486 (55.8)	385 (44.2)				
Diverticulosis				0.013	0.909		
Yes	51	28 (54.9)	23 (45.1)				
No	865	482 (55.7)	383 (44.3)				
Numbers at first colonoscopy				0.163	0.686		
< 3	580	320 (55.2)	260 (44.8)				
≥ 3	336	190 (56.5)	146 (43.5)				
Concomitance with protruding adenoma(s) at first colonoscopy		127.154			< 0.001		
No	280	234 (83.6)	46 (16.4)			1	
Yes	636	276 (43.4)	360 (56.6)			7.759 (4.420-13.618)	< 0.001
Bowel preparation				45.773	< 0.001		
Good	757	460 (60.8)	297 (39.2)			1	
Poor	159	50 (31.4)	109 (68.6)			4.389 (2.314-8.352)	< 0.001
Adenoma-related							
Size				122.706	< 0.001		
≥ 10 mm	172	159 (92.4)	13 (7.6)			1	
6-9 mm	391	202 (51.7)	189 (48.3)			9.239 (4.306-19.824)	< 0.001
< 6 mm	353	149 (42.2)	204 (57.8)			19.613 (8.984-42.822)	< 0.001
Location				74.571	< 0.001		
Rectum	184	152 (82.6)	32 (17.4)	6.81	0.009[1]	1	
Left colon	322	175 (54.3)	147 (45.7)			2.866 (1.623-5.062)	< 0.001
Right colon	410	183 (44.6)	227 (55.4)			3.259 (1.819-5.838)	< 0.001
Advanced adenoma				113.850	< 0.001		
Yes	205	181 (88.3)	24 (11.7)				
No	711	329 (46.3)	382 (53.7)				
Pathologic classification				74.745	< 0.001		
Tubular	745	366 (49.1)	379 (50.9)				
Tubulovillous and villous	151	132 (87.4)	19 (12.6)				
Serrated	20	12 (60.0)	8 (40.0)				
Procedure-related							
Proficiency				28.18	< 0.001		
> 1000 cases	585	363 (62.1)	222 (37.9)			1	
500-1000 cases	234	109 (46.6)	125 (53.4)			2.219 (1.397-3.525)	0.001
< 500 cases	97	38 (39.2)	59 (60.8)			3.003 (1.568-5.754)	0.001
Specialty				6.86	0.009		
Gastroenterologist	739	427 (57.8)	312 (42.2)				
Non-gastroenterologist	177	83 (46.9)	94 (53.1)				
Operative mode				4.862	0.027		
One-person technique	721	415 (57.6)	306 (42.4)				
Two-person technique	195	95 (48.7)	100 (51.3)				
Withdrawal time				6.069	0.014		
≥ 6 min	254	158 (62.2)	96 (37.8)			1	
< 6 min	662	352 (53.2)	310 (46.8)			1.958 (1.276-3.006)	0.020

[1]The comparison between the left and right colon.

Table 4 "Per-adenoma" miss rates and risk factors in detection of advanced flat adenomas during colonoscopy *n* (%)

Factor	Total (*n* = 205)	Diagnosed (*n* = 181)	Missed (*n* = 24)	χ^2 test	*P* value
Size				17.796	< 0.001
< 10 mm	33	22 (66.7)	11 (33.3)		
≥ 10 mm	172	159 (92.4)	13 (7.6)		
Location				11.838	0.003
Rectum	68	67 (98.5)	1 (1.5)	1.127	0.288[1]
Left colon	36	32 (88.9)	4 (11.1)		
Right colon	101	82 (81.2)	19 (18.8)		
Pathologic classification				0.025	0.874
Tubular	57	50 (87.7)	7 (12.3)		
Tubulovillous and villous	148	131 (88.5)	17 (11.5)		
Age (yr)				1.157	0.282
< 60	98	89 (90.8)	9 (9.2)		
≥ 60	107	92 (86.0)	15 (1.4)		
Bowel preparation				13.815	< 0.001
Good	162	150 (92.6)	12 (7.4)		
Poor	43	31 (72.1)	12 (27.9)		
Proficiency				1.078	0.583
> 1000 cases	135	120 (88.9)	15 (11.1)		
500-1000 cases	55	49 (89.1)	6 (10.9)		
< 500 cases	15	12 (80.0)	3 (20.0)		
Specialty				6.167	0.013
Gastroenterologist	156	146 (93.6)	10 (6.4)		
Non- gastroenterologist	49	35 (71.4)	14 (28.6)		
Operative mode				2.272	0.132
One-person technique	161	145 (90.1)	16 (9.9)		
Two-person technique	44	36 (81.8)	8 (18.2)		
Withdrawal time					
≥ 6 min	145	129 (89.0)	16 (11.0)	0.217	0.641
< 6 min	60	52 (86.7)	8 (13.3)		

[1]Comparison between the left and right colon.

relation coefficient was 0.516 (*P* < 0.001).

DISCUSSION

The present study revealed that colorectal flat adenomas are very common in patients undergoing colonoscopy. Moreover, flat adenomas are more frequently seen in the Oriental population than in the Western population[26]. However, this type of adenoma is frequently missed in clinical practice, especially when the flat adenoma is concomitant with a protruding adenoma(s). In the present study, the "per-adenoma" flat adenoma miss rate (44.3%) is significantly higher than the overall adenoma miss rate (20.9%). It is also significantly higher than that of the protruding adenoma (15.1%). These results are in line with those of previous reports[6,27].

Moreover, multivariate logistic regression analysis showed that many factors including patient-related, adenoma-related, and procedure-related ones influenced the miss rates for colorectal flat adenomas. In the present study, age was shown to be an independent risk factor affecting the detection of flat adenomas. Patients older than 60 years had a much higher miss rate than those younger than 60 years. This finding is consistent with a previous study, which showed that miss rate was higher in patients with older age in both univariate and multivariate analyses[16].

The quality of bowel preparation also affects the detection of flat adenomas. Sufficient bowel preparation is a prerequisite for a better view of flat adenomas[28]. Poor bowel preparation places more impacts on the detection of flat adenomas than protruding adenomas[7]. Our present study showed that poor bowel preparation was closely correlated with a higher miss rate for flat adenomas, which was as high as 68.6%. Correspondingly, the miss rate for advanced flat adenomas also increased significantly with poor preparation. When the bowel is poorly prepared, mucus and chymes released from the small intestine could easily accumulate in the cecum and ascending colon, and thus, it is even more difficult to clean out these parts of the colon than the left half of the colon. Previous studies have shown that the quality of bowel preparation, especially in the right colon, can be improved by changing the cleaning methods so as to increase the flat adenoma detection rates[7,29].

It has been known that protruding adenomas frequently co-exist with flat adenomas[4]. In the present study, co-existence was observed in approximately one fourth (514/2093) of patients with colorectal adenomas. Previous studies have demonstrated that co-existence of concomitant protruding adenomas is an independent risk factor affecting the detection rate for flat adenomas[30]. The present study demonstrated that the miss rate in patients with flat adenomas and concomitant protruding adenomas was significantly higher than those with flat adenomas only or those with protruding adenomas

only. For the first time, co-existence with concomitant protruding adenomas was identified as an independent risk factor affecting the miss rate for flat adenomas. We postulate that once the protruding adenomas are detected, the less apparent flat adenomas could be easily neglected by the colonoscopist because the detection of the protruding adenoma represents a positive endoscopic diagnosis, which is usually considered by the colonoscopist to be the cause of the indication (*e.g.*, clinical symptoms or signs) for colonoscopy. Also, the numbers of adenomas were more than three in most patients with flat and concomitant protruding adenomas in a previous observation[4]. Our study showed that 44.3% patients with flat and concomitant protruding adenomas had more than three adenomas at the first colonoscopy, which is in agreement with the previous observation.

It has been suggested that the number of colorectal adenomas identified at the initial colonoscopy is positively associated with the miss rates for overall adenomas[6,27]. However, in the present study, there was no significant association between the numbers of adenomas detected at the first colonoscopy and the miss rates for flat adenomas in both univariate and multivariate analyses. The discrepancy between the present study and previous studies suggests the difference in the risk factors for the miss rates between flat adenomas and overall adenomas, in terms of the number of adenomas identified at the initial colonoscopy. In other words, the adenoma numbers at the first colonoscopy is associated with the miss rate for overall adenomas, but not with the miss rate for flat adenomas. Specifically, the adenoma number of ≤ three at the first colonoscopy is associated with a lower miss rate for overall adenomas, compared with the number of more than three[6,26]. However, there was no association between the adenoma number at the first colonoscopy and AMR for flat adenomas. Even if the adenoma number at the first colonoscopy is less than three, AMR for flat adenomas is still very high and similar to that in those patients with more than three adenomas at the first colonoscopy.

The present study revealed that adenoma location and size significantly affected the miss rates for this type of adenoma. We also found that the miss rate was much higher for adenomas < 10 mm than those ≥ 10 mm and for flat adenomas in the right colon than those in the left colon, which is different from the miss rate for overall adenoma observed in a previous study[6,16]. The reasons for the discrepancy are considered as follows: (1) Unlike protruding adenomas, flat adenomas are majorly localized at the right colon[13,31]; and (2) the colonic pouch at the right colon is deep and big, and thus it is difficult to detect the flat adenoma inside the deep colonic pouch. Therefore, it is essential to take measures such as training in fold exploration, specification of right colon withdrawal time, and examination of the right colon twice to improve the detection rate for adenomas at the right colon. The miss rates for flat adenomas in the left and right colon were significantly higher than that in the rectum, clearly indicating that the miss rate for flat adenoma in the colon

is much higher than that in the rectum. Our study also revealed that the proportions of histologically villous adenomas and high-grade dysplastic adenoma in patients with flat adenomas were significantly higher than those in patients with protruding adenomas, which was consistent with previous observations[4,5,31]. In the present study, we reported an incidence (1.1%) of flat depressed adenomas, which is similar to that (1.4%) observed by Nicolás-Pérez *et al*[30], indicating that the incidence of flat depressed adenomas is very low. Based on previous findings[4,5,30], it is conceivable that the flat depressed adenomas may have a great tendency to develop dysplasia or even neoplasia. Indeed, flat depressed adenomas appeared to be more associated with high-grade dysplasia as observed in the present study. However, the number of cases with this type of adenomas in the present study was small and thus further investigation with a larger number of cases with this particular type of adenoma is needed. In addition, we found that the miss rate for advanced adenomas was slightly higher, albeit not statistically significantly, in the right colon than that in the left colon. This finding is in agreement with previous observations that flat adenomas including those located at the right colon have a higher tendency to become malignant than the protruding adenoma[4,5,31]. Thus, reduction of the miss rate for flat adenomas will help to decrease the occurrence of the colon cancer, especially in the right colon.

In the present study, proficiency of colonoscopists and the withdrawal time of colonoscopy were identified as independent risk factors affecting the miss rates for flat adenomas, which is consistent with previous observations on the miss rates for the overall adenoma[32-34]. Specifically, the miss rates significantly decreased in patients undergoing colonoscopy performed by more experienced and knowledgeable operators. It has been shown that the staining endoscopy technique is very helpful in improving the detection of flat adenomas by conventional white light colonoscopy[13,35]. Therefore, we recommend that less-experienced endoscopists learn this technique as soon as possible. Currently, the withdrawal time is required to be kept for more than six minutes according to the colonoscopy guidelines in order to guarantee the quality of colonoscopy[34], as the withdrawal time of more than six minutes can carefully observe colonic mucous and effectively reduce the miss rate for flat adenomas. Our study further supports that the withdrawal time of more than 6 min can reduce the miss rate and improve the detection of flat adenomas.

The background of colonoscopists may affect the quality of colonoscopy. Bressler and colleagues[36] suggested that colonoscopy by an internist or family physician was an independent risk factor for new or missed CRC. In addition, the protective effects of colonoscopy on the right and left colon when colonoscopy was performed by gastroenterologists are similar. However, the protective effect against colon cancer was less on the right colon than on the left colon if colonoscopy was performed by non-gastroenterologists[37]. In the present

study, univariate analysis showed that flat adenomas, as well as advanced flat adenomas, in cases operated by gastroenterologists were less likely missed than those by non-gastroenterologists. However, multivariate analysis did not reveal this finding. Thus, we postulate that the impact of colonoscopy by non-gastroenterologists on the miss rate for flat adenomas may be demolished if the colonoscopists are well-trained and the quality of colonoscopy improved. In addition, our study showed that there was no difference in the miss rate between colonoscopies performed by one person and those by two persons, in the multivariate analysis, which is consistent with a previous observation that the operative mode of colonoscopy performed by either single or double operators had no significant influence on the quality of colonoscopy[38].

We further analyzed the association between the potential risk factors and the miss rates for advanced flat adenomas, and found that the proficiency of endoscopists, mode of operation, and withdrawal time were not associated with the miss rates for advanced flat adenomas. The main reasons are that the advanced adenomas are usually large, or the villous structure on the colonic surface is more easily recognized. Therefore, the miss rates could be low even if the endoscopists lack experience, and the withdrawal time is shorter than the conventional standards. It should be stated that only univariate analysis was used to determine the association due to a limited number of patients with advanced flat adenomas, and thus studies with large sample sizes are needed to further determine the risk factors for advanced flat adenomas.

Kahi *et al*[39] suggested that it would be reasonable to consider that improved detection of nonpolypoid lesions by thorough and high-quality colonoscopy will result in improved overall adenoma detection rates. On the contrary, in the largest study of flat adenoma detection, Reinhart *et al*[25] demonstrated only a poor correlation between the overall adenoma detection rate and the detection rate for flat adenomas ($r = 0.24$). Thus, they did not support the recommendation of adding the flat adenoma detection rate to the widely accepted adenoma detection rate in clinical practice[40]. So far, there has been no report on the correlation between the miss rate for the overall adenomas and that for flat adenomas. The present study, from the perspective of AMR analysis, showed a better correlation ($r = 0.516$), compared with Reinhart's study regarding the adenoma detection rates. Therefore, we postulate that, on the routine colonoscopy, the missed adenomas are proportional to the missed flat adenomas, and thus, measures should be taken to avoid missing flat adenomas.

There were some limitations in this study. For example, due to the retrospective nature of the study, the withdrawal time was counted by the average time of an endoscopist to perform a negative colonoscopy instead of the actual time in each patient; nevertheless, this method has been applied previously[18]. In addition, some auxiliary techniques such as chromoscopy and high-definition colonoscopy were excluded from this study. Studies have

shown that applications of these techniques are able to reduce the miss rate for flat adenomas[10,14]. However, for the routine colonoscopy, these techniques may not be required as previous studies have shown similar miss rates for colorectal adenomas[9,15]. It should be mentioned that the endoscopic morphologic classification of flat adenomas may be important in predicting post resection recurrence. However, the present study did not focus on this issue and, due to the small number of flat depressed adenomas (only 10 out of 916 flat adenomas), any analyzed results based on this number may not be clinically meaningful, and thus a well-designed study specifically targeting this issue is warranted.

In conclusion, the miss rate for flat adenomas during colonoscopy is high. Patient's age, concomitant protruding adenomas, bowel preparation, size and location of adenomas, proficiency of the colonoscopist, and withdrawal time are factors affecting the "per-adenoma" AMR for flat adenomas. These findings have significant clinical implications in helping clinicians to detect and treat flat adenomas and thus prevent the development of CRC, and reduce the incidence, morbidity and mortality of CRC.

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COMMENTS

Background
Colorectal cancer (CRC) is the most common malignant tumor and the second leading cause of cancer-related deaths in the world. CRC mainly originates from colorectal adenomas. It has been reported that flat colorectal adenomas have a greater tendency to develop into severe dysplasia and carcinoma than protruding adenomas

Research frontiers
Flat adenomas are frequently missed during colonoscopy. However, the risk factors that influence their miss rates are unclear.

Innovations and breakthroughs
The study aimed to determine the miss rate for colorectal flat adenomas during colonoscopy and the risk factors. The "per-patient" and "per-adenoma" adenoma miss rates (AMR) for overall adenomas and flat adenomas, and patient-, adenoma-, and procedure-related risk factors potentially associated with the "per-adenoma" AMR for flat adenomas were determined. Chromoscopy and high-definition colonoscopy were not taken under consideration in the study.

Peer review
This is an interesting paper on the risk factors associated with missed flat adenomas, which provides a contribution to the current knowledge of this topic.

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RETROSPECTIVE STUDY

New strategy during complicated open appendectomy: Convert open operation to laparoscopy

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Abstract

AIM: To introduce a new strategy during complicated open appendectomy - converting open operation to laparoscopy.

METHODS: We retrospectively reviewed databases at two institutions between October 2010 and January 2013, identifying 826 patients who had undergone complicated appendectomy for histologically confirmed acute or chronic appendicitis. They included 214 complicated appendectomies: 155 lengthened-incision open appendectomies (LIA group) and 59 open appendectomies with conversion to laparoscopy (OACL group).

RESULTS: A total of 214 patients with complicated appendectomies were included in the study, including 155 cases of LIA and 59 cases of OACL. No major complication leading to death occurred in the study. Patient characteristics of the two groups were similar. Several parameters showed a significant difference between the two groups. For the OACL *vs* LIA groups they were, respectively: incision length (3.8 ± 1.4 cm *vs* 6.2 ± 3.5 cm, $P < 0.05$); time to flatus recovery (2.3 ± 0.6 d *vs* 4.2 ± 0.8 d, $P < 0.05$), drainage rate (61.0% *vs* 80.0%, $P < 0.05$); pain level (3.6 ± 1.8 *vs* 7.2 ± 2.4, $P < 0.05$); hospital stay (5.1 ± 2.7 d *vs* 8.7 ± 3.2 d, $P < 0.05$); complication rate (8.5% *vs* 14.7%, $P < 0.05$). Other factors showed no significant differences.

CONCLUSION: Lengthened-incision open appendectomy increases the incidence of complications and prolongs the hospital stay. Conversion of open to laparoscopic appendectomy is feasible and efficient in complicated cases. It decreases the rate of incisional and abdominal infections, allows faster return of bowel movements, and shortens the hospital stay.

Key words: Complicated appendectomy; Open; Laparoscopy; Conversion; Complication

Core tip: In the present paper, we introduce a new strategy during complicated open appendectomy: convert to laparoscopy. It is an effective and safe technique when comparing the length of incisions. Moreover, in this report, we describe some techniques applied in laparoscopic appendectomy to minimize complications.

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INTRODUCTION

Laparoscopic appendectomy has rapidly developed since Semm[1] published an article reporting the first complete

removal of the appendix *via* laparoscopic surgery in 1983 and Schreiber[2] performed the first laparoscopic appendectomy in a patient with acute appendicitis in 1987. Although open appendectomy remains the gold standard worldwide for treating complicated appendiceal disease[3-7], the laparoscopic technique has improved and appendectomies are being increasingly performed by laparoscopy. Laparoscopy converted to an open procedure is a conventional strategy during complicated appendectomies. A much larger incision than that needed for laparoscopy is routinely applied in those procedures, resulting in a high rate of complications, such as incisional infections, which in turn cause a prolonged hospital stay and high level of pain for the patient[8-10]. Since October 2010, we have attempted to convert open appendectomies to laparoscopic procedures during complicated cases instead of lengthening the incision. This new strategy has produced good clinical results.

MATERIALS AND METHODS

Patients and methods

From October 2010 to January 2013, a total of 826 appendectomies (519 open, 307 laparoscopic) were performed at The Second Affiliated Hospital Zhejiang University School of Medicine. Having to choose between a lengthened incision or conversion to laparoscopy during the appendectomy procedure was considered a "complicated" appendectomy. Based on this definition, 214 complicated appendectomies had been performed, including 155 lengthened-incision appendectomies (LIA group) and 59 open appendectomies that were converted to laparoscopy (OACL group). The indication for appendectomy in the study was acute or chronic appendicitis. The initial strategy for all 214 cases was open appendectomy. Because of difficulty during the procedures, however, they were changed to either LIA or OACL. The outcomes of the two approaches were compared on an intention-to-treat basis.

The mean ages of the OACL and LIA groups were 29.6 ± 14.2 years *vs* 30.1 ± 16.7 years, respectively. The patients' characteristics and perioperative data were recorded before and after surgery. The characteristics are shown in Table 1.

Surgical technique for conversion of open to laparoscopic appendectomy

Among the 214 open appendectomies, 59 operations were converted to laparoscopic appendectomy (OACL group) because it was difficult to complete the procedure through a normal incision. The remaining 155 other appendectomies continued as open procedures but were performed after lengthening the incision (LIA group).

All of the OACL patients were converted to general endotracheal anesthesia. A 10-mm trocar was placed in the open incision, after which the incision was closed by suture to make sure no gas could pass through. We used a conventional three-port technique. After the first tro-

Table 1 Patient characteristics for the open appendectomy converted to laparoscopy and lengthened-incision appendectomy groups

Variable	OACL group (n = 59)	LIA group (n = 155)	P value
Age, yr, mean ± SD	29.6 ± 14.2	30.1 ± 16.7	NS
Sex, female/male, n	32/27	75/80	NS
WBC counts (× 10⁹/L), mean ± SD	14.8 ± 4.2	13.9 ± 3.4	NS
C reactive peptide, U/L, mean ± SD	18.8 ± 3.9	20.1 ± 4.5	NS
Localized abdominal tenderness n (%)	48 (81.4)	119 (76.8)	NS
Preoperative scale of pain (range 1-10), mean ± SD	2.9 ± 1.5	2.7 ± 1.8	NS

OACL: Open appendectomy converted to laparoscopy; LIA: Lengthened-incision appendectomy; WBC: White blood cell.

Figure 1 Positions of trocars.

car was placed (Figure 1), CO_2 gas was input to keep the pressure between 12 and 15 mmHg. A supraumbilical port was created for the camera, and another was placed medial to the left anterosuperior iliac spine. Figure 2 shows laparoscopic visualization of the appendix during OACL.

The following procedures were nearly the same for both the OACL and LIA groups. During the operation, three strategies were applied for three situations: (1) the appendix was in the right upper abdomen, but all of it could be found easily; (2) the base of the appendix could be identified easily, but the tip was difficult to identify; and (3) a mass was present because the appendix was perforated, which made it difficult to distinguish the appendix from adjacent intestine. In the first situation, harmonic shears (Harmonic Scalpel; Ethicon EndoSurgery, Somerville, NJ, United States) were used to separate the appendix by dividing the mesoappendix. As the appendix was separated completely, it was ligated at its base and cut. It could then be removed in a specimen bag through the large incision. In the second situation, we performed a retrograde appendectomy by separating and then ligating the base of the appendix. We then used harmonic shears to divide the appendix from its base to its tip. In the third situation, we performed a submucosal appendectomy, a technique which has been introduced by Hannan and Hoque[11]. An incision was made on the

Figure 2 Appearance of the appendix during the open appendectomy converted to laparoscopy procedure.

Table 2 Perioperative data for the open appendectomy converted to laparoscopy and lengthened-incision appendectomy groups *n* (%)

Variable	OACL group (*n* = 59)	LIA group (*n* = 155)	*P* value
Operative time, min, mean ± SD	45.6 ± 17.2	43.8 ± 16.1	NS
Length of incision, cm, mean ± SD	3.8 ± 1.4	6.2 ± 3.5	< 0.05
Flatus, d, mean ± SD	2.3 ± 0.6	4.2 ± 0.8	< 0.05
Drain placed	36 (61.0)	124 (80.0)	< 0.05
Scale of pain (range 1-10), mean ± SD	3.6 ± 1.8	7.2 ± 2.4	< 0.05
Days of drainage, d, mean ± SD	2.1 ± 1.1	2.7 ± 1.6	NS
Reoperation	0 (0)	2 (1.3)	NS
Hospital stay, d, mean ± SD	5.1 ± 2.7	8.7 ± 3.2	< 0.05
Complications	4 (6.8)	23 (14.7)	< 0.05
Intra-abdominal abscess	1 (1.7)	7 (4.5)	< 0.05
Wound infection	2 (3.4)	11 (7.1)	< 0.05
Ileus	1 (1.7)	1 (0.6)	NS
Fecal fistula	0 (0)	2 (1.3)	NS
Bleeding	0 (0)	2 (1.3)	NS
Histopathology			NS
Acute	40 (67.8)	93 (60.0)	
Phlegmonous	8 (13.6)	18 (11.6.)	
Gangrenous or perforated	9 (15.3)	28 (18.1)	
Periappendicular abscess	2 (3.5)	16 (10.3)	

OACL: Open appendectomy converted to laparoscopy; LIA: Lengthened-incision appendectomy.

antimesenteric wall of the appendix, and the mucosal sleeve was pulled out, leaving the muscular wall in place. The base of the tube was then ligated flush with the cecum and divided distally. The muscular tube was left alone. Normal saline was used to clean the peritoneal cavity. Closed suction drain tubes were placed in most of the cases that were in the second and third situations described earlier.

Postoperative management

Liquids were allowed 6 h after the operation. Feeding was allowed after flatus recovery in the majority of cases of both groups. A few patients with a fragile appendiceal base had their feeding restricted until complete recovery of bowel movements. The drainage tube was removed only when there was no collection in the drainage bag. There were some rare cases of drainage continuing after discharge from the hospital. In such cases, the drainage tube was removed once there was no collection and no fever.

Statistical analysis

Numerical data were expressed as the mean ± SD. Statistical significance was evaluated by Student's *t*-test or the χ^2 analysis between the two groups with SPSS 17.0 for Windows software (SPSS Inc., Chicago, IL, United States). *P* < 0.05 was considered statistically significant.

RESULTS

All 59 patients in the OACL group underwent surgery that was successful. No reconversion to open surgery or reoperation was necessary. In contrast, 2 of 155 LIA patients required reoperation because of incisional bleeding and intra-abdominal bleeding, respectively. All patients were ultimately discharged in good health.

The patient characteristics, including sex, age, C-reactive peptide, and scale of pain showed no significant difference between the two groups. The factors that were significantly different between the two groups were the following (with respective values for the OACL *vs* LIA groups): length of incision 3.8 ± 1.4 cm *vs* 6.2 ± 3.5 cm; time to flatus recovery 2.3 ± 0.6 d *vs* 4.2 ± 0.8 d; rate of drainage 61.0% *vs* 80.0%; scale of pain 3.6 ± 1.8 *vs* 7.2 ± 2.4; hospital stay 5.1 ± 2.7 d *vs* 8.7 ± 3.2 d; complication rate 6.8% *vs* 14.7%. Other perioperative data were not significantly different for the two groups. Among the complications, abdominal abscess and incision infection were found more often in the LIA group than in the OACL group. Two cases of fecal fistula occurred in the LIA group and contributed to prolonged hospital stays, but the occurrence rate was not different in the two groups. The perioperative data are shown in Table 2.

All patients were followed up at the clinic or by telephone interview for 2 to 22 mo, during which time no major complications occurred.

DISCUSSION

Laparoscopy offers more advantages than the open technique in terms of postoperative outcomes, including less pain, fewer complications, and faster recovery. Unlike laparoscopic cholecystectomy, which became the "gold standard" for removing gallbladder disease, laparoscopic appendectomy still has some controversial issues. This is especially true in regard to complicated appendectomies[12-15]. As the instrumentation improves and experience increases, some surgical centers consider laparoscopic surgery the first choice for treating acute appendicitis. However, open appendectomy is still accepted as the gold standard and is widely performed, and it remains first choice for appendicitis in many institutions world-wide. Over a 3-year period during 2010-2013, a total of 519 open and 307 laparoscopic appendectomies were performed in our institution. The open technique is considered reliable and easily performed, with a low

incidence of morbidity[16]. Therefore, the conventional appendectomy strategy is laparoscopy with conversion to open surgery or open appendectomy directly. When difficulties arise during open appendectomy, however, a larger incision may be needed to search for and then divide the appendix. The problem is that a large incision and confused anatomy may lead to a high complication rate[17-20]. Beginning in October 2010, we attempted to convert open appendectomies to laparoscopy when it was difficult to find the appendix and/or to separate it. We achieved good results when we applied a new strategy - converting the open procedure to laparoscopic appendectomy - and compared it to simply lengthening the incision to complete the open operation.

The OACL has the same advantages as laparoscopic appendectomy (LA). There are differences between OACL and conventional three-port LA, however. The position of the trocar on the right abdomen for OACL is at the McBurney point, whereas for LA it is at a higher position, which leads to some differences in the technique. The short distance between the trocar and the appendix during OACL makes it difficult to manipulate the instruments. To solve this problem, the trocar at the McBurney point is sometimes used for the camera. The other difference is the method for removing the appendix. It is removed through a McBurney incision during OACL but through a supraumbilical incision during LA.

Retrograde and submucosal appendectomies have been performed by both open and laparoscopic methods when difficulty is encountered during a procedure[21]. A subserosal appendix has been described with extensive serosal adhesions, which generally cover the body and tip of the appendix but not the base[22]. Retrograde appendectomy is useful for this situation. As the base of the appendix is divided, clips (Lapro-Clip, Covidien, Mansfield, MA, United States; or Hem-o-lock, Weck Closure Systems, Research Triangle Park, NC, United States) are used to ligate it, after which it is cut. Harmonic shears are used to separate the appendix from the base to the tip. The key to the maneuver is that harmonic shears can go beyond the wall of the cecum and approach the appendix. Submucosal appendectomy was reported to be an effective technique for most cases of complicated appendicitis[11]. Once the appendix is identified, the serosal and muscular layers are incised by hook cautery, taking care not to perforate the mucosa (unless it was perforated already). In the case of perforation, divisions begin at the perforation. A metal aspirator with a blunt tip is useful for separating layers between muscular tissue and mucosa. The division continues (as above) until the mucosal tube of the appendix is separated completely, leaving the serosal and muscular tube with an incision on its surface. The procedure is easily performed. Minor bleeding might occur but is easy to control by hook cautery or harmonic shears. The advantages of submucosal laparoscopic appendectomy are as follows: (1) it is not necessary to divide the appendix and cecum, which avoids perforating the wall of the cecum; (2) it is not necessary to divide the mesoappendix, which contains the appendiceal artery and vein, which could easily be injured; and (3) it is not necessary to separate the appendix from adjacent intestine and peritoneum. For some cases, the combined technique is feasible.

Harmonic shears are much more useful than hook cautery during OACL. They are effective for the first two of the three situations mentioned above - appendix on the right upper abdominal but easily found; base of appendix easily identified but not the tip; a mass owing to appendiceal perforation, making it difficult to distinguish it from adjacent intestine. The mesoappendix and adhesions could be divided by harmonic shears directly without ligation, which decreases the incidence of bleeding and shortens the operative time. Ligation of the mesoappendix is sometimes uncertain during open appendectomy and causes a threat. In one patient who underwent LIA abdominal bleeding occurred because of uncertain ligation, and reoperation was necessary to stop it. In our experience, harmonic shears are not as important in the third situation as in the other two situations. A metal aspirator would be helpful for aspiration and for blunt separation.

Incisional infections and intra-abdominal abscesses are common complications after appendectomy[23,24]. It was recently reported that these two complications are less common after laparoscopy than after open appendectomy[23,25-27]. We found the same results for OACL *vs* LIA: the intra-abdominal abscess and incisional infection rates were 1.7% and 3.4%, respectively, after OACL, which were significantly lower than those for LIA. Conversion to laparoscopy in our patients allowed direct visualization during peritoneal toileting. The cleaner peritoneal cavity led to a lower occurrence of intra-abdominal abscesses. Compared with LIA, the OACL procedure was completed under closed incisions (in the peritoneal cavity), which contributed to a lower chance of incisional contamination and certainly a lower incidence of incisional infection.

Fecal fistula and ileus are serious complications of appendectomy, although they occur at low rates[28,29]. These two complications delay bowel movement recovery and prolong hospital stay. Early physical movement and drainage removal are effective measures to prevent ileus. Restricted feeding for patients whose appendiceal base was fragile helps prevent and/or decreases the seriousness of the fecal fistula. In our study, the incidences of fecal fistula and ileus in the OACL and LIA groups were low, with no significant differences between the groups.

A long incision increases the patient's pain and is a poor-healing wound. Postoperative pain was correlated with recovery of bowel movements, which was one of the reasons why time to flatus recovery was shorter in the OACL group than in the LIA. Feeding was started after bowel movement recovery, which enhanced wound healing in the OACL group and led to a shorter hospital stay.

Laparoscopic appendectomy can be the first choice in most cases of appendicitis. OACL is a safe, feasible procedure when difficulty is encountered during open appendectomy. It contributes to a low rate of complications and is in accord with the concept of minimally invasive surgery. It provides a new strategy for dealing with the open complicated appendectomy. Skilled laparoscopic technique is necessary for the OACL procedure.

COMMENTS

Background
Appendicitis is one of the most common diseases, with open appendectomy the gold standard treatment. During complicated appendectomy, a large incision is often necessary, increasing the possibility of complications. With improved laparoscopic technique, laparoscopic appendectomy has become the first choice for appendectomy. However, laparoscopic appendectomy for complicated appendicitis remains controversial and open operation is still applied world-wide.

Research frontiers
Laparoscopic appendectomy has been widely used for treatment of appendicitis. New techniques including single-port laparoscopic and natural orifice transluminal endoscopic surgery have been applied for appendectomy. Clinical outcomes and effects between traditional laparoscopy and new techniques for appendectomy have been compared.

Innovations and breakthroughs
Laparoscopy converted to an open procedure is a conventional strategy during complicated appendectomies. A much larger incision than that needed for laparoscopy is routinely applied in those procedures, resulting in a high rate of complications. The strategy is a reversal conception. Converting open to laparoscopy instead of lengthening incision was used for complicated appendectomy.

Applications
It is feasible and efficient to convert open to laparoscopic appendectomy when the procedure is difficult to perform by normal incision, which decreases the rate of incision infection and abdominal infection, and results in faster bowel movement and shortened hospital stay.

Peer review
It is a well written manuscript, which described a unique concept. The strategy is helpful for centers where open appendectomies as first choice are being done.

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RETROSPECTIVE STUDY

Model based on γ-glutamyltransferase and alkaline phosphatase for hepatocellular carcinoma prognosis

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Abstract

AIM: To determine the prognostic value of alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) for hepatocellular carcinoma (HCC) .

METHODS: We analyzed the outcome of 172 HCC patients who underwent liver resection. Receiver operating characteristic (ROC) curve analysis was performed to determine the cut-off value of ALP and GGT. Then, preoperative risk factors for survival were evaluated by multivariate analysis. Based on the significant factors, a prognostic score model was established.

RESULTS: By ROC curve analysis, ALP > 120 U/L and GGT > 115 U/L were considered elevated. Overall survival (OS) and tumor-free survival (TFS) for patients with elevated ALP and GGT were significantly worse than for patients with ALP and GGT within the normal range. Multivariate analysis showed that the elevated levels of ALP, GGT and tumor size were independent prognostic factors. Giving each positive factor as a score of 1, we established a preoperative prognostic score model. The 5-year OS for patients with a score of 0, 1, 2 and 3 were 84.0%, 45.9%, 44.1% and 0%, respectively, while the TFS was 80.6%, 40.0%, 38.8% and 0%, respectively. When combining patients with scores of 1 and 2 into the middle risk group, and patients with scores of 0 and 3 into the low-risk and high-risk groups, respectively, different outcomes would be significantly distinguished by the risk groups.

CONCLUSION: Elevated ALP and GGT levels were risk predictors in HCC patients. Our prognostic model might vary the outcomes of patients from different risk groups.

Key words: Alkaline phosphatase; γ-Glutamyltransferase; Prognosis; Hepatocellular carcinoma

Core tip: To determine the optimal cut-off value of alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) to predict hepatocellular carcinoma (HCC) prognosis after liver resection, and to establish a scoring model, we analyzed the outcome of 172 HCC patients who underwent liver resection. Receiver operating characteristic curve analysis was performed to determine the cut-off value of ALP and GGT. Preoperative risk factors for survival were evaluated by multivariate analysis. Based on the significant factors, a prognostic scoring model

was established. Our model might affect the outcome of patients in different risk groups, and was superior to the traditional risk markers.

INTRODUCTION

Hepatocellular carcinoma (HCC), the fifth most common cancer worldwide, is rarely detected early and is usually fatal within months of diagnosis[1]. Liver resection remains the gold standard for patients with resectable HCC that develops in the setting of normal liver substance. However, most patients with HCC have diseased liver parenchyma, especially HBV-related cirrhosis in China, and resection in this population is more fraught, with the potential for more complications[2].

Thus, a balance for choice of therapy is urgently needed prior to treatment, such as liver transplantation, transcatheter hepatic arterial chemoembolization, and radiofrequency ablation. Yet, these choices are mostly based on traditional guidelines such as the Milan Criteria (single nodule ≤ 5 cm or two or three nodules ≤ 3 cm)[3] and the University of California, San Francisco (UCSF) Criteria (single nodule ≤ 6.5 cm, or two or three nodules with the largest nodule ≤ 4.5 cm and the total tumor burden ≤ 8 cm)[4]. Great suspicions were raised about these criteria, because they solely rely on inaccurate preoperative imaging findings such as tumor number and tumor size, but neglect the essential features of tumors such as carcinoembryonic antigen and α-fetoprotein (AFP). However, although AFP is frequently used to predict post-hepatectomy outcomes in patients with HCC, contradictory results have been reported from different studies, and the predictive accuracy was far from satisfactory[5,6].

Building a scoring model combining the imaging findings and serum parameters to predict the prognosis of HCC patients undergoing liver resection is useful in guiding us to choose the best treatment. Many trials have focused on exploring the prognostic markers of HCC in patients undergoing liver resection, such as serum miRNAs and other potential gene markers, however, they have had either unsatisfactory results, or were confined to laboratory experiments, which were not implementable clinically[7-9].

Serum liver enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and γ-glutamyltransferase (GGT), are routinely tested in patients. These enzymes are com-

monly elevated in patients with liver diseases and thus may reflect the status of liver injury[10]. Of the liver enzymes, ALP and GGT have long been recognized to play potential roles in the diagnosis of cancer. For instance, Xu *et al*[11] systemically analyzed the isoenzymes of GGT, and reported that GGT could be applied as an additional marker for HCC, valuable not only for the diagnosis of clinical HCC, but also for the detection of small or subclinical HCC. Hann *et al*[12] studied the associations of liver enzymes with the risk of HCC in HBV-infected patients, and found that compared to patients with normal baseline GGT, those with elevated GGT exhibited a significantly increased HCC risk with a hazard ratio of 2.60. Lopez *et al*[13] reported that raised ALP level in the presence of normal bilirubin was more often a feature of HCC than benign liver diseases, although the specific mechanism was not clear.

However, these studies were mainly focused on the diagnostic roles of ALP and GGT. Few studies have systematically explored the prognostic roles of these liver enzymes. In the current study, we sought to evaluate the effects of ALP and GGT on the long-term prognosis of patients with HCC undergoing liver resection. In addition, we tried to combine these serum markers such as ALP and GGT, and tumor characteristics such as tumor number and tumor size, to establish a scoring model consisting of comprehensive features of tumors to predict better the prognosis of HCC.

MATERIALS AND METHODS

Patient selection

Prospectively collected data in our unit (First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, China) were reviewed retrospectively. We enrolled 172 HCC patients underwent liver resection with complete follow-up during the 10-year period from December 2002 to July 2012. For this study, we included those patients who met all the following criteria: (1) patients were diagnosed with only HCC, but with no concomitant intrahepatic cholangiocarcinoma, or any other malignancies, to eliminate the confounding effects from disease etiology; (2) patients had serum liver enzymes (ALP and GGT) and AFP measured simultaneously at study entry, making the baseline analyses comparable; (3) liver resection was performed on the resectable HCC; and (4) patients had a minimum follow-up time of 1 year from the study entry point.

Data collection

Patient baseline and clinical data, including age, sex, liver enzymes such as ALP and GGT, serum AFP, HBV infection, HBV-DNA level, HCV infection, preoperative imaging data (tumor size, number, and invasion), surgery procedure records, and tumor pathology were recorded. Other synthetic liver function was also assessed, such as total bilirubin, albumin and international normalized ratio (INR), to evaluate the Child classification of every patient. Liver resection specimens were assessed by two
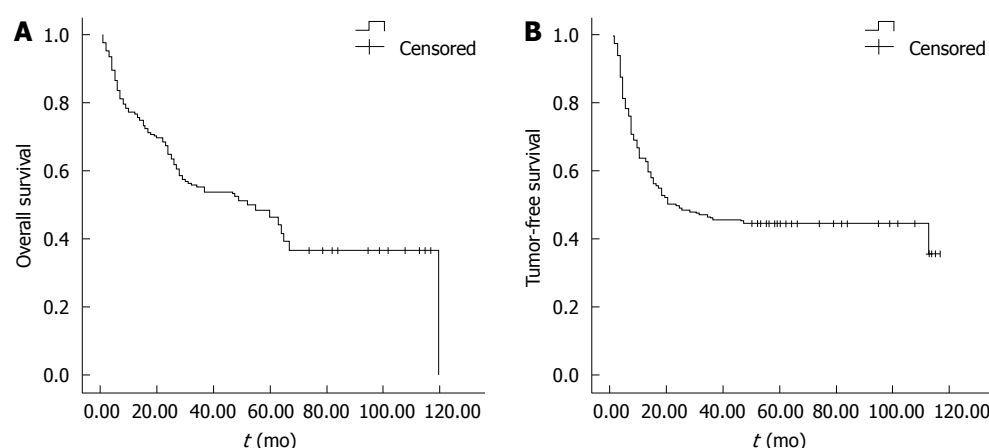
Figure 1 Kaplan-Meier curves of (A) overall survival, and (B) progression-free survival, for the whole 172 patients underwent liver resection enrolled in this study.

independent liver pathologists, blinded to all patient demographics and clinical outcomes. All patients gave written informed consent to this study, and approval for the study was obtained from the Institutional Review Board, which conformed to the standards of the Declaration of Helsinki.

Surgery and postoperative management

A systematic examination was performed to exclude peritoneal metastases. The resection techniques principally involved were either anatomical or nonanatomical according to the patients' preoperative liver function and tumor anatomical status. Hepatic pedicle and celiac lymph nodes were removed for frozen section histological examination. Systematic, intraoperative hepatic ultrasonography was performed to detect additional nodules or portal thrombosis. After resection, the macroscopic features of the tumor, including size, number of tumors, portal vein invasion, and hepatic vein invasion, were recorded. Histological examinations of microscopic vascular invasion as well as satellite lesions were examined. Postoperative management included symptomatic therapy if any surgical complications occurred, such as bleeding, infection, or hypoalbuminemia.

Follow-up

After liver resection, patients were followed every 3 mo in the first year, every 4 mo in the second year, and every 6 mo thereafter. Imaging with computed tomography or magnetic resonance imaging was obtained for each patient on every follow-up visit, along with liver function analysis and serum AFP level. Tumor recurrence was diagnosed based on the combined findings of these clinical examinations. Patients who developed recurrence were treated with repeat hepatic resection whenever possible, or otherwise with transcatheter arterial embolization.

Statistical analysis

All data were analyzed by the SPSS version 19.0 software (SPSS, Chicago, IL, United States). Comparisons between the two groups were done using a *t*-test for continuous

data and the χ^2 test for categorical data, with $P < 0.05$ considered significant. The survival curves were constructed by the Kaplan-Meier method and compared by the log-rank test, stratified by GGT and ALP, with the cutoff points determined by the receiver operating characteristic (ROC) curve analysis. Multivariate Cox regression analysis was performed to evaluate the prognostic significance of the variables in predicting overall survival (OS). Results are given as mean ± SD.

RESULTS

Patient demographics and outcomes

One hundred and thirty-nine patients (80.8%) were men and 33 (19.2%) women. The mean age was 53.5 years (range: 24-80 years). We were able to determine Child-Pugh classification from the available clinical records in all the enrolled patients, based on which, 160 cases were classified as Class A and 12 as Class B. No Class C patients were enrolled in this study, because Class C disease is a contraindication for hepatic resection in our department. Altogether, 87 patients died during follow-up. Of the 76 patients who developed tumor recurrence, 46 (60.5%) developed recurrence within 1 year and 69 (90.7%) within 2 years after surgery. Mean follow-up time was 2.91 years (range: 0.1-10 years). The 1-, 3- and 5-year OS for all patients included in this study were 74.1%, 54.4% and 46.6%, respectively (Figure 1).

ROC curves showed the cut-off value for elevated ALP and GGT

ROC curve analysis revealed an optimal cutoff of 121 U/L for ALP and 117 U/L for GGT in terms of predicting survival. As to ALP, the area under the ROC curve (AUC) was 0.631, with a 95%CI of 0.547-0.714 (Figure 2A), while the AUC for GGT was 0.643 (95%CI: 0.560-0.725) (Figure 2B). A cut-off value of 121 presented a sensitivity of 41.4% and a specificity of 85.9% for ALP, and a cut-off value of 117 presented a sensitivity of 39.1% and a specificity of 85.9% for GGT. In order to be utilized clinically, we chose a cutoff value of 120 for
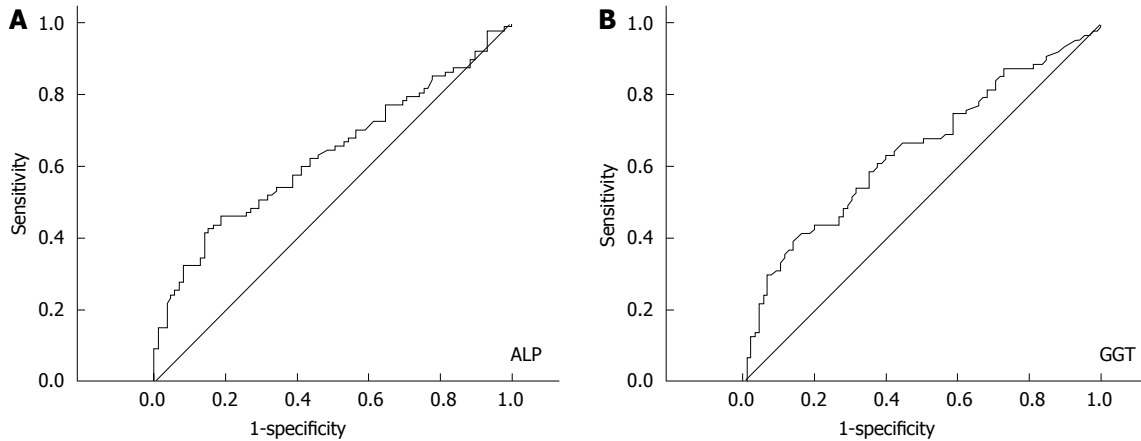
Figure 2 Receiver operating characteristic curves to discriminate 172 hepatocellular carcinoma patients with different prognosis by the appropriate cutoff values of alkaline phosphatase (A) and γ-glutamyltransferase (B). ALP: Alkaline phosphatase; GGT: γ-glutamyltransferase.

Table 1 Preoperative factors affecting the overall survival and tumor-free survival of hepatocellular carcinoma patients

Category	Subcategory	Overall survival			Tumor-free survival		
		Univariate analysis	Multivariate analysis	HR (95%CI)	Univariate analysis	Multivariate analysis	HR (95%CI)
Gender	Male (139)	0.935			0.916		
	Female (33)						
Age	≥ 60 yr (55)	0.090			0.233		
	< 60 yr (117)						
HBV	Yes (121)	0.044	0.012	0.556	0.084		
	No (51)			(0.353-0.878)			
HCV	Yes (8)	0.637			0.485		
	No (164)						
Cirrhosis	Yes (59)	0.321			0.141		
	No (113)						
ALP	> 120 U/L (50)	< 0.001	0.008	1.866	< 0.001	0.002	1.973
	≤ 120 U/L (122)			(1.176-2.960)			(1.283-3.034)
GGT	> 115 U/L (48)	< 0.001	0.030	1.674	0.001	0.676	
	≤ 115 U/L (124)			(1.050-2.668)			
AFP	≥ 400 ng/mL (66)	0.085			0.001	0.017	1.685
	< 400 ng/mL (106)						(1.099-2.583)
Tumor characteristics							
Size	≥ 5 cm (114)	< 0.001	< 0.001	4.472	< 0.001	< 0.001	4.315
	< 5 cm (58)			(2.328-8.590)			(2.299-8.099)
Number	> 1 (32)	0.143			0.218		
	= 1 (140)						
Invasion	Yes (54)	0.319			0.379		
	No (118)						
Lymphnode metastasis	Yes (6)	0.001	0.242		< 0.001	0.009	3.149
	No (166)						(1.329-7.462)

ALP: Alkaline phosphatase; GGT: γ-glutamyltransferase; AFP: α-fetoprotein; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

ALP and 115 for GGT, without significant impairment of the diagnostic accuracy of ALP and GGT.

Factors associated with OS and tumor-free survival in HCC patients

Univariate and multivariate analysis of factors affecting OS and tumor-free survival (TFS) of HCC patients are shown in Table 1. Univariate analysis revealed that, tumor size, lymph-node metastasis, HBV infection, ALP and GGT were preoperative prognostic predictors of poor OS. Multivariate regression analysis was performed on all preoperative factors that were significant in univariate

analysis, revealing tumor size, HBV infection, ALP and GGT as independent factors associated with OS (Table 1).

With regard to TFS, again, on univariate analysis, the presence of lymph-node metastasis, tumor size, ALP, GGT and AFP level were correlated with TFS. By further multivariate regression analysis, the presence of lymph-node metastasis, tumor size, ALP and AFP level were confirmed to be independent factors associated with the TFS of HCC patients.

In these preoperative factors, our multivariate analysis showed that the hazard ratio (HR) of HBV infection for OS was 0.556, which was contrary to the accepted
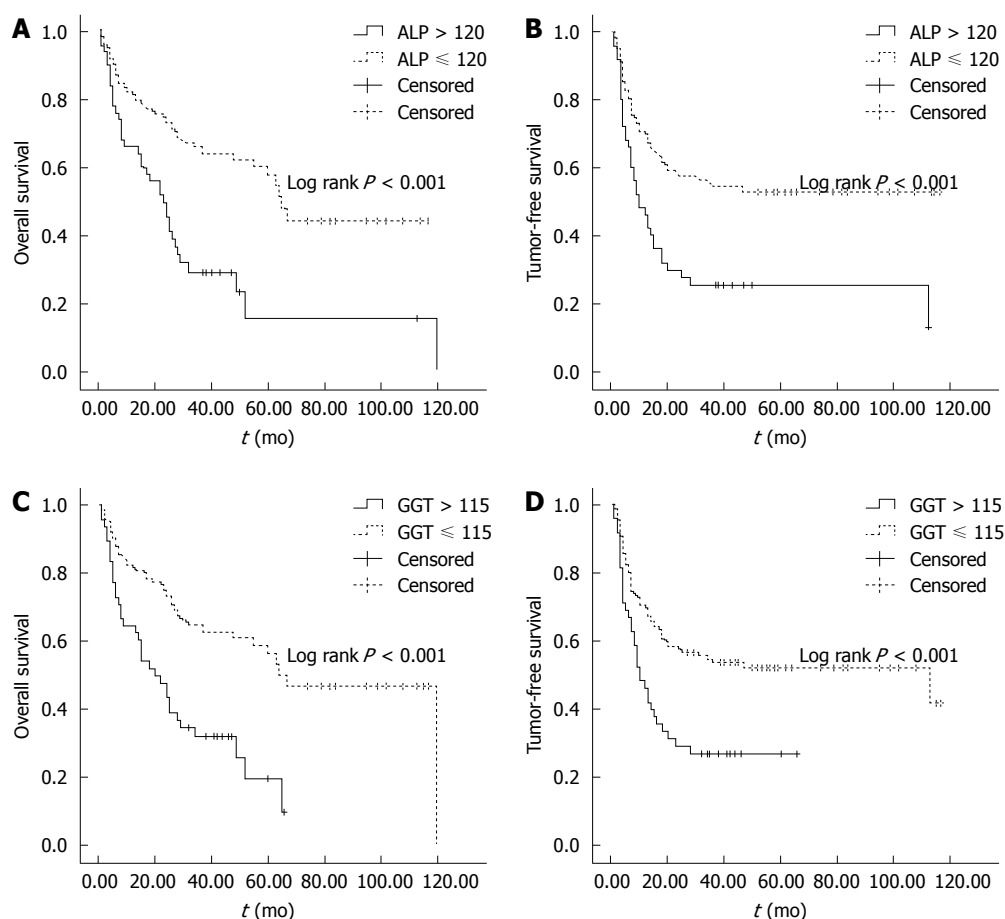
Figure 3 Impact of alkaline phosphatase and γ-glutamyltransferase on the overall (A and C) and tumor-free survival (B and D) following surgical resection, as classified by the cutoff value of alkaline phosphatase, and γ-glutamyltransferase, respectively. ALP: Alkaline phosphatase; GGT: γ-glutamyltransferase.

consensus that HBV infection is a risk factor for prognosis of HCC patients, so we excluded this factor in the further analysis. We also excluded lymph-node metastasis and AFP level in the following analysis, because they were not independent factors in OS, which was more important in predicting the prognosis of HCC patients who underwent liver resection. In summary, we chose the easily accessible indices such as ALP, GGT, and tumor size as preoperative predictive factors, which were all independently associated with OS, and played important roles in the regression analysis of TFS.

Elevated ALP and GGT were associated with worse prognosis in HCC patients

ALP was elevated at 50 in 172 patients (29.1%) and GGT was elevated at 48 in 172 patients (27.9%). For both ALP and GGT, a significant difference was observed in the OS and TFS between patients with normal and elevated levels. In terms of ALP, the 1-, 3- 5-year OS and TFS in patients with normal ALP level were 81.1%, 65.8% and 60.3%, and 70.0%, 54.1% and 52.8%, respectively, compared with 66.2%, 29.1% and 15.6% (OS) and 45.4%, 25.2% and 25.2% (TFS) in patients with elevated ALP (*P* < 0.05, Figure 3A, B). In terms of GGT, the 1-, 3- and 5-year OS and TFS in patients with normal GGT level

were 80.7%, 61.7% and 56.4%, and 70.0%, 53.2% and 51.8%, respectively, compared with 64.5%, 32.9% and 19.1% (OS), and 45.3%, 25.9% and 25.9% (TFS) in patients with elevated GGT (*P* < 0.05, Figure 3C, D).

The baseline characteristics in patients with normal or elevated ALP and GGT are shown in Table 2. There were no significant differences in sex, age, HBV, HCV, cirrhosis, AFP, and tumor characteristics such as tumor number, invasion, and lymph-node metastasis between the different groups according to ALP and GGT. The only significantly different factor in both the ALP and GGT groups was tumor size (*P* < 0.05), which might partially explain the varied outcomes of prognosis in patients with different ALP and GGT levels.

Construction of the preoperative prognostic scoring model

Inspired by the preoperative prognostic score published by Wang *et al*[14] in HCC patients who underwent transplantation, we established a preoperative model using the three preoperative factors, namely, ALP, GGT, and tumor size, which were found to be significant by multivariate regression analysis.

We defined each positive factor as a score of 1, and accordingly divided the patients into four groups, name-

Table 2 Comparison of baseline characteristics of patients with different alkaline phosphatase, and γ-glutamyltransferase

Factors	ALP > 120 (*n* = 50)	ALP ≤ 120 (*n* = 122)	*P* value	GGT > 115 (*n* = 48)	GGT ≤ 115 (*n* = 124)	*P* value
Gender	40	99	0.835	40	99	0.671
Age	19	36	0.286	18	37	0.365
HBV	34	87	0.714	35	86	0.712
HCV	2	6	1.000	3	5	0.687
Cirrhosis	23	36	0.051	20	39	0.215
AFP	17	49	0.493	21	45	0.386
Tumor characteristics						
Size	39	75	0.050	42	72	< 0.001
Number	10	22	0.830	11	21	0.387
Invasion	9	45	0.018	16	38	0.719
Lymphnode metastasis	3	3	0.358	4	2	0.052

ALP: Alkaline phosphatase; GGT: γ-glutamyltransferase; AFP: α-fetoprotein; HBV: Hepatitis B virus; HCV: Hepatitis C virus.
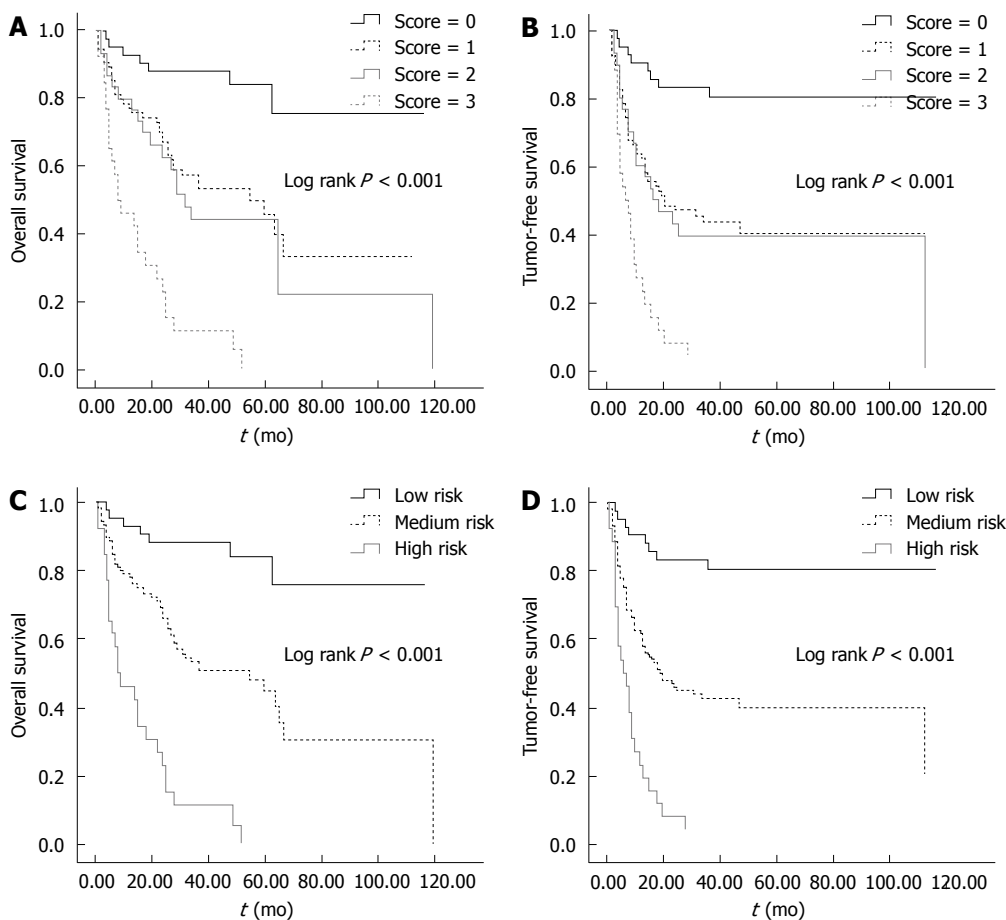
Figure 4 Varied outcomes of hepatocellular carcinoma patients as classified by different prognostic scores (A and B) and different degrees of risk (C and D).

ly, a score of 0, 1, 2 or 3. Varied outcomes in OS and TFS stratified by different scores are shown in Figure 4. The 5-year OS for patients with a score of 0, 1, 2 or 3 was 84.0%, 45.9%, 44.1% or 0%, respectively ($P < 0.05$, Figure 4A). With respect to TFS, the 5-year survival for patients with a score of 0, 1, 2 or 3 was 80.6%, 40.0%, 38.8% or 0%, respectively ($P < 0.05$, Figure 4B). Although the OS and TFS of the patients with a score of 3 decreased sharply compared with those with a score < 3, and patients with a score of 0 had the longest survival, no significant difference was seen between the patients with a score of 1 or 2. Therefore, we combined the patients with a score of 1 and 2 as medium risk, and defined the patients with a score of 0 and 3 as low and high risk, respectively. Thus, the postoperative prognosis could be easily predicted by the Kaplan-Meier curves stratified by high, medium and low risks ($P < 0.05$, Figure 4C, D).

Table 3 Univariate Cox regression analysis of prognostic scoring model, alpha-fetoprotein level, tumor size and the presence of cirrhosis

	P value	HR	95%CI
Low risk	-	-	-
Middle risk	< 0.001	4.250	1.927-9.370
High risk	< 0.001	15.56	6.181-39.175
AFP	0.085	1.454	0.950-2.227
Cirrhosis	0.321	0.790	0.495-1.259
Tumor size	< 0.001	5.139	2.722-9.703

AFP: α-fetoprotein.

Predictive value of the prognostic scoring model

The predictive value of the prognostic scoring model, compared with the traditional prognostic markers such as AFP level, tumor size, and presence of cirrhosis, by univariate Cox proportional hazards analysis, is summarized in Table 3. The prognostic score was superior to the traditional prognostic markers of AFP and cirrhosis, and more accurate than tumor size as prognostic markers, with medium and high risks having HRs of 4.250 and 15.560, respectively.

DISCUSSION

Among appropriately selected patients with HCC, liver resection provides excellent outcomes, with a 5-year survival rate of 70%[15]. However, even under strict screening, 68% of HCC patients still develop tumor recurrence in 5 years after liver resection[15]. According to our results, the 1-, 3- and 5-year OS rates for all patients included in this study were 74.1%, 54.4% and 46.6%, respectively; a little lower than the survival rates reported by Roayaie *et al*[15], which could be explained by the fact that patients in their study with tumor size < 2 cm. In contrast, our patients had an average tumor size of 6.95 cm. Thus, to develop novel noninvasive biomarkers for patients suitable for liver resection is urgently needed, to avoid tumor recurrence and surgical complications.

Despite the fact that several markers have been intented to guide prognosis in HCC, few were of significant prognostic value, or too inconvenient to implement clinically. Inflammatory markers have long been linked with malignancy, and Virchow first observed leukocytes in neoplastic tissue in the mid-1800s, suggesting an important role for inflammation in the development of malignancies[16]. Inflammation contributes to the development of at least 15% of all cancers, especially of the digestive system[17]. For example, patients with HBV infection experience chronic inflammation, which increases the risk of liver cancer[18].

With respect to hepatitis virus, as one of the most common etiologies of HCC, the estimated risk of HCC is 15-20-fold higher in persons infected with hepatitis virus than in uninfected persons. HBV is predominant in the east and HCV in the west, therefore, carriers of both viruses have a substantial risk of HCC-related death.

However, we failed to demonstrate HBV or HCV as an independent risk factor; probably due to the development of antiviral therapy in recent years, or because of the small number of patients in our cohort.

ALP and GGT are liver enzymes that are routinely tested clinically for liver function evaluation. ALP is a hydrolase enzyme, which is present in all tissues throughout the entire body, but particularly concentrated in the liver, bile duct, kidney, bone, and placenta[19]. Clinically, as a stable serum marker, high levels of serum ALP are indicative of hepatic or bile-tract-associated disease. The ALP level also increases if bone formation occurs, because ALP is a byproduct of osteoblast activity. In addition, ALP has already been included in the Chinese University Prognostic Index, an HCC staging system that assigns a score of 3 when ALP is > 200 IU/L, indicating the potential roles of ALP in predicting the prognosis of HCC patients[20].

GGT is a nearly ubiquitous epithelial enzyme, which initiates the degradation of extracellular glutathione and its conjugates and correlates with biotransformation, nucleic acid metabolism, and tumorigenesis[21]. Moreover, elevation of serum GGT was detected in a series of clinical conditions other than hepatobiliary disorders, including pancreatic disease, myocardial infarction, renal failure, and diabetes[10]. With respect to cancer risk, significant associations have been reported between elevated GGT and increased risk of cancer. Furthermore, a previous study showed that GGT was a potential predictor in liver-specific diseases in both HBV patients and the general population in western countries[22].

However, preoperative liver function tests, specifically ALP and GGT, and their values in long-term follow-up of HCC patients have seldom been systematically explored. We extensively evaluated the association between the liver enzymes ALP and GGT and prognosis of HCC patients undergoing liver resection. We found significant elevation of ALP and GGT levels before surgery, which independently predicted prognosis in HCC patients. In addition, this effect was also significantly increased when we combined the two serum markers with tumor size, and we successfully constructed a preoperative prognostic scoring model.

In this study, we systematically explored the cut-off value of ALP and GGT by using ROC curve analysis in predicting prognosis in HCC patients. We found that the cut-off value of ALP and GGT was 121 U/L and 117 U/L, respectively, which were a little higher than the those reported by others when utilized in prediction of prognosis[23,24]. Utilizing the ALP and GGT values with relatively high sensitivity and specificity in multivariate regression analysis, our results showed that ALP, GGT and tumor size were independent prognostic predictors of poor OS and TFS. Further analysis showed patients with elevated ALP and GGT had significantly higher risks of death and tumor recurrence by Kaplan-Meier analysis, indicating the potential predictive roles of ALP and GGT in the prognosis of HCC patients undergoing liver resec-

tion. Although the specific mechanism is still unclear, there are several possible hypotheses.

Previous studies have shown that ALP is a differentiation marker for embryonic and other stem cells derived from the bone and adipose tissue. In addition, ALP was found to indicate cancer cell proliferation in nucleolar localization in an electron microscopic cytochemistry study[25]. Cancer cells showed higher ALP activity in the nucleolus and changes in localization during the cell cycle, which revealed the roles of ALP in tumor proliferation and progression, besides its common correlation with cholestasis and hepatitis. With respect to GGT, its impact on tumorigenesis might be mediated by the functions of the oxidative stress pathways in cellular responses[26]. There is extensive evidence that GGT and glutathione (GSH), the degradation of which is catalyzed by GGT, can cooperatively generate free radicals and thus lead to lipid peroxidation. On the other hand, lipid peroxidation is significantly implicated in the tumorigenesis of many malignancies including HCC, which might also partially explain the GGT-HCC association[27,28].

Although we demonstrated the prognostic roles of GGT and ALP in predicting the prognosis of HCC, however, there was no significant correlation with respect to Child score, when we explored the potential mechanisms of GGT and ALP in cancer prognosis. We speculated that it was the tumor features represented by GGT and ALP, rather than the traditional values of GGT and ALP in liver function reserve, that affect OS of HCC patients.

Based on multivariate analysis, we further established a simple prognostic model with an AUC of 0.745. When we divided the patients into different groups by giving each positive factor as a score of 1, the 5-year OS for patients with a score of 0, 1, 2 or 3 was 84.0%, 45.9%, 44.1% or 0%, respectively, while the TFS was 80.6%, 40.0%, 38.8% or 0%, respectively. Considering the similarities in prognosis of patients with scores of 1 and 2, we combined these patients into the medium-risk group, while patients with scores of 0 and 3 were allocated into the low-risk and high-risk group, respectively. On this basis, varied outcomes were significantly divided by risk groups. When compared with the traditional prognostic markers such as AFP level, tumor size and presence of cirrhosis, the predictive value of the prognostic model was significantly more accurate by univariate analysis, with HRs of the medium- and high-risk groups of 4.25 and 15.56, respectively[6,29,30].

It is worth noting that although elevated ALP and GGT might predict prognosis of HCC in some way, we should not be totally dependent on these markers. Many other factors affecting ALP and GGT, such as hepatobiliary disorders and bone formation, could impair the accuracy of prognostic prediction. In addition, HBV or HCV infection, tumor number, and lymph-node metastasis, might also affect the prognosis of HCC, although the effect was not significant in our study, probably due to the limited number of patients[31]. Thus, further studies are still needed to confirm and update our preoperative scoring model to predict the prognosis of HCC.

COMMENTS

Research frontiers
Although α-fetoprotein is frequently used to predict post-hepatectomy outcomes in patients with hepatocellular carcinoma (HCC), contradictory results have been reported from different studies, and the predictive accuracy was far from satisfactory. In this respect, building a scoring model combining the imaging findings and serum parameters to predict the prognosis of HCC patients undergoing liver resection is useful in choosing the best treatment.

Research frontiers
Alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) have long been recognized to play potential roles in the diagnosis of cancer. However, few studies have systematically explored the prognostic roles of these liver enzymes. In the current study, the authors sought to evaluate the effects of ALP and GGT on the long-term prognosis of patients with HCC undergoing liver resection.

Innovations and breakthroughs
Recent reports have highlighted the importance of ALP and GGT in cancer prognosis. The authors of the manuscript successfully demonstrated the prognostic roles of ALP and GGT in HCC. Furthermore, they combined these serum markers such as ALP and GGT, and tumor characteristics such as tumor number and tumor size, to establish a scoring model consisting of comprehensive features of tumors to better predict the prognosis of HCC.

Applications
By establishing the novel prognostic scoring model consisting of comprehensive features of tumors, this study may represent a future strategy for cancer prediction in the follow-up of patients with hepatocellular carcinoma.

Terminology
Serum liver enzymes such as ALP, and GGT, are routinely tested in clinical patients. These enzymes are commonly elevated in patients with liver diseases and thus may reflect the status of liver injury. Recently, ALP and GGT have also been recognized to play potential roles in the diagnosis of cancer, highlighting the potential prognostic roles in hepatocellular carcinoma.

Peer review
The goal of this clinical study was to evaluate the predictive values of alanine aminotransferase and γ-glutamyltransferase on the prognosis of patients with HCC and liver resection. To this aim, 172 HCC patients were enrolled with complete follow-up for 10-years and it was concluded that elevated ALP and GGT levels were risk predictors for this population. This is a well-conducted study and well-written paper with interesting information to the readers. The authors have provided a very even handed and documented discussion why these biochemical markers are valuable tools for addressing this important and relevant question.

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RETROSPECTIVE STUDY

Living donor liver transplantation does not increase tumor recurrence of hepatocellular carcinoma compared to deceased donor transplantation

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Abstract

AIM: To compare the recurrence-free survival (RFS) and overall survival (OS) of hepatitis B virus (HBV)-positive hepatocellular carcinoma (HCC) after living donor liver transplantation (LDLT) and deceased donor liver transplantation (DDLT).

METHODS: We retrospectively collected clinical data from 408 liver cancer patients from February 1999 to September 2012. We used the chi-squared test or Fisher's exact test to analyze the characteristics of LDLT and DDLT. Kaplan-Meier analysis was used to compare the RFS and OS in HCC.

RESULTS: Three hundred sixty HBV-positive patients (276 DDLT and 84 LDLT) were included in this study. The mean follow-up time was 27.1 mo (range 1.1-130.8 mo). One hundred eighty-five (51.2%) patients died during follow-up. The 1-, 3-, and 5-year RFS rates for LDLT were 85.2%, 55.7%, and 52.9%, respectively; for DDLT, the RFS rates were 73.2%, 49.1%, and 45.3% (P = 0.115). The OS rates were similar between the LDLT and DDLT recipients, with 1-, 3-, and 5-year survival rates of 81.8%, 49.5%, and 43.0% *vs* 69.5%, 43.0%, and 38.3%, respectively (P = 0.30). The outcomes of HCC according to the Milan criteria after LDLT and DDLT were not significantly different (for LDLT: 1-, 3-, and 5-year RFS: 94.7%, 78.7%, and 78.7% *vs* 89.2%, 77.5%, and 74.5%, P = 0.50; for DDLT: 86.1%, 68.8%, and 68.8% *vs* 80.5%, 62.2%, and 59.8% P = 0.53).

CONCLUSION: The outcomes of LDLT for HCC are not worse compared to the outcomes of DDLT. LDLT does not increase tumor recurrence of HCC compared to DDLT.

Key words: Hepatocellular carcinoma; Living donor; Deceased donor; Liver transplantation; Hepatitis B virus

Core tip: Whether there is a higher tumor recurrence for living donor liver transplantation (LDLT) than for deceased donor liver transplantation (DDLT) for hepatocellular carcinoma (HCC) has recently become a subject of debate. Our results suggest that LDLT does not increase the tumor recurrence of HCC compared to DDLT. The recurrence-free survival and long-term survival times of LDLT for HCC are higher than those of DDLT.

Xiao GQ, Song JL, Shen S, Yang JY, Yan LN. Living donor liver transplantation does not increase tumor recurrence of hepatocellular carcinoma compared to deceased donor transplantation. *World J*

INTRODUCTION

Liver transplantation (LT) is an ideal treatment for hepatocellular carcinoma (HCC) because it can completely clear a tumor in the liver and improve the patient's liver function. Many studies have demonstrated that the outcomes of HCC patients according to the Milan criteria (single tumor ≤ 5 cm in size or ≤ 3 tumors each ≤ 3 cm in size, and no macrovascular invasion) or the University of California, San Francisco (UCSF) criteria (single tumor ≤ 6.5 cm, or 3 or fewer nodules with the largest lesion ≤ 4.5 cm and a total tumor diameter ≤ 8 cm, without vascular invasion) are positive[1-5]. Most researchers suggest that the long-term outcomes of LT are better than those of hepatectomy for HCC with Milan or UCSF criteria[2,4]. Nonetheless, LT is greatly limited by the shortage of available livers. Many HCC patients on waiting lists have died before a live graft could become available. The idea of using living donor liver grafts for orthotopic LT started in 1966 and 1969[6,7]. It took more than 20 years for the idea to materialize in clinical practice[8].

Living donor living transplantation (LDLT) is considered an alternative to deceased donor living transplantation (DDLT). Many researchers have suggested that the recurrence-free survival (RFS) and overall survival (OS) rates of patients are similar for LDLT and DDLT[9-17]. However, some investigators have indicated that the RFS and OS rates of LDLT are worse compared to the rates after DDLT[18-23]. In this study, we aim to compare the prognoses of HCC patients after LDLT and DDLT at our transplantation center, followed by a comparison of the outcomes of HCC after LDLT and DDLT according to the Milan, UCSF, and Hangzhou criteria.

MATERIALS AND METHODS

Patient selection

We obtained the patient data from the China Liver Transplant Registry (CLTR) database. The demographic and clinical data of 408 liver cancer patients who underwent LT at our center from February 1999 to September 2012 was retrospectively collected, with preoperative demographic, clinical, and laboratory data for all patients being recorded. Systemic imaging was employed within 1 week before surgery. The pathological data for the explanted livers were considered the gold standard for tumor assessment. Vascular invasion and tumor differentiation were also assessed by pathology. Patients who were not hepatitis B virus (HBV)-positive, were not diagnosed with HCC by pathology, were younger than 18 years-old, or had died within 1 mo after transplantation were excluded from this study. This study was approved by the Institutional Review Board of the West China Hospital of Sichuan University in Sichuan Province. We obtained informed written consent from all patients according to the Declaration of Helsinki of the World Medical Association.

Surgical procedures

A modified technique for adult-to-adult LDLT was used at our LT center[24]. We used the surgical technique of the anterior approach for liver resection for recipients. First, we dissected the first porta area and disconnected the left and right branches of the hepatic artery, biliary duct, and portal vein. Second, we blocked the retrohepatic inferior vena cava and suprahepatic vena cava and then removed the recipient liver. The anterior approach provides a "no-touch" technique in resecting the liver tumor, decreasing the chance of tumor rupture and metastasis. After liver resection, 5-fluorouracil solution was used to lavage the peritoneal cavity.

The retro-hepatic portion of the inferior vena cava was removed along with the liver, and the piggyback technique (the recipient's inferior vena cava being preserved) was employed for LDLT patients. In the early stages for DDLT, the standard technique without the use of a venovenous bypass was used at our center. The piggyback technique was implemented for most of the DDLT patients.

Postoperative treatment

After surgery, the patients received immunosuppressive drugs, including corticosteroids, cyclosporine, or tacrolimus with or without mycophenolate mofetil. The blood dosages of cyclosporine, tacrolimus, and mycophenolate mofetil were maintained at low levels. In general, corticosteroids were withdrawn after 3 mo of treatment. HBV immunoglobulins or antivirus drugs, such as lamivudine, adefovir, telbivudine, and entecavir, were administered in HBV-positive LT patients after operation[25].

Patient follow-up

After transplantation, the patients were followed-up with outpatient visits or by telephone, and every year we invited the patients who had received LT to visit our center. The recipients received routine blood examinations, α-fetoprotein (AFP) tests, and chest X-ray examinations every month in the first year. In the first half of the second year, the patients received these examinations once every 2 mo. In the following years, the patients underwent these examinations every 3-6 mo, or when necessary. When necessary, we took abdominal computed tomography (CT), abdominal magnetic resonance imaging, chest CT, head CT, and bone scans of the patients. Any suspicious lesions in the liver or lungs of the recipients were biopsied, if deemed necessary. Brain and bone pain, as well as progressive growth of bone, were recorded. The date of tumor recurrence was considered as the time that the AFP level began to rise after tumor recurrence had been confirmed. If patients had tumor recurrence, we recorded the time and administered the appropriate treatment. If

Table 1 Comparison of the demographic and clinicopathological data of hepatitis B virus-related hepatocellular carcinoma patients after living donor liver transplantation and deceased donor liver transplantation

Variables	LDLT	DDLT	P value
	(*n* = 84)	(*n* = 276)	(2-tailed)
Gender (F/M)	6/78	29/247	0.36
Age-yr (mean)	44.3	47.3	0.06
Age, yr (< 60/ ≥ 60)	79/5	238/38	0.05
BMI (< 24/24-27/ ≥ 27)	57/19/8	185/65/26	0.98
Child-Pugh (A/B/C)	43/34/7	137/119/20	0.89
Meld score (≤ 10/10-20/> 21)	47/33/2	150/92/19	0.25
AFP (μg/L) (< 400/ ≥ 400)	37/43	141/135	0.45
Preoperative adjuvant therapy (Y/N)	17/67	101/175	0.005[1]
Tumor No. (≤ 3/> 3)	62/18	206/50	0.56
Largest Tumor Size (≤ 5/5-9/> 9)-cm	40/24/16	128/65/73	0.35
Total tumor size (≤ 5/5-9/> 9)-cm	28/21/31	77/41/91	0.46
Vascular invasion (Y/N)	20/64	97/179	0.05
Milan criteria (Y/N)	22/58	69/199	0.75
UCSF criteria (Y/N)	28/52	84/184	0.54
Hangzhou criteria (Y/N)	39/41	112/145	0.42
HBV-DNA-copies/ml (< 1.00E + 03/> 1.00E + 03)	32/52	76/131	0.83
Differentiation (1-2/3-4)	32/11	114/70	0.13

[1]Significant *P* value. UCSF: University of California, San Francisco; HBV: Hepatitis B virus; LDLT: Living donor liver transplantation; DDLT: Deceased donor liver transplantation; BMI: Body mass index; AFP: α-fetoprotein.

the patient died, we recorded the time and cause of death.

Statistical analysis

We used SPSS v17.0 to analyze the data. The independent sample *t*-test, Pearson's χ^2 test, and Fisher's exact test were used to analyze the differences in the demographic and clinical data from patients after LDLT and DDLT. Kaplan-Meier survival analysis was used to analyze the RFS and OS rates of the HCC patients. The statistical data were expressed as mean ± SD. The confidence interval quoted area was 95%; statistically significant differences were defined as $P < 0.05$.

RESULTS

Patient demographics and outcomes

The data for patients who underwent LT came from the CLTR database. The patients were regularly followed up to December 2012. Of all 408 liver cancer patients who received LT at our medical center from February 1999 to September 2012, 48 patients were excluded from this study; eighteen were not HCC patients, eighteen patients died within 1 month after transplantation, ten were not HBV-positive, and two patients were younger than 18 years-old. This left 360 patients to be included from this study. Of the included patients, 84 (23.3%) received LDLT, and 276 (76.7%) received DDLT. Among the 360 patients, there were 35 (9.7%) women and 325 (90.3%) men. The mean age of the HCC patients who received

LT was 46.6 ± 9.86 years. The mean follow-up time of all patients was 2.22 years (range: 1.1-10.7 years).

The demographic and clinical data of all LDLT and DDLT patients are shown in Table 1. There was a statistically significant difference in the preoperative adjuvant therapy. There were no significant differences in terms of recipient gender; age; body mass index; Child-Pugh score; Meld score; AFP level; tumor number; largest tumor size; total tumor size; vascular invasion; adherence to the Milan, UCSF, or Hangzhou criteria; HBV-DNA level; or tumor differentiation (Table 1).

According to our analysis, the percentage of HCC patients who received the preoperative adjuvant therapy was higher in patients after DDLT than LDLT. Of the 118 patients who received preoperative adjuvant therapy, 83 (70.3%) received transcatheter arterial chemoembolization therapy only, 18 (15.3%) patients radiofrequency ablation treatment only, 11 (9.3%) underwent hepatectomy only, and 6 (5.1%) patients received more than two treatment methods. The analysis suggested that preoperative adjuvant therapy had no impact on the RFS and OS between DDLT and LDLT.

Outcome of the HCC patients after LDLT and DDLT

Of all 360 patients included in this study, the median wait times for LDLT and DDLT were 0.9 and 1.6 mo, respectively. A total of 138 (38.3%) patients had tumor recurrence, and 198 (55.0%) patients died during follow-up. The 1-, 3-, and 5-year RFS rates of the patients in our study were 76.2%, 50.9%, and 47.2%, respectively, while the 1-, 3-, and 5-year OS rates were 72.5%, 4.5%, and 40.0%, respectively. The 1-, 3-, and 5-year RFS rates were 85.2%, 55.7%, and 52.9% for LDLT *vs* 73.2%, 49.1%, and 45.3% for DDLT ($P = 0.12$). The 1-, 3-, and 5-year OS rates were 81.8%, 49.5%, and 43.0% for LDLT *vs* 69.1%, 43.0%, and 38.3% for DDLT ($P = 0.30$). There were no significant differences in the RFS and OS rates between LDLT and DDLT (Figure 1).

We divided all HCC patients who underwent LDLT and DDLT according to the Milan, UCSF, and Hangzhou criteria into 6 categories. We then compared the RFS and OS rates of these categories. The outcomes are shown in Figure 2. The 1-, 3-, and 5-year RFS rates according to the Milan criteria were 94.7%, 78.7%, and 78.7% for LDLT *vs* 89.2%, 77.5%, and 74.5% for DDLT ($P = 0.50$). The 1-, 3-, and 5-year RFS rates according to the UCSF criteria were 95.5%, 82.6%, and 82.6% for LDLT *vs* 88.0%, 74.0%, and 71.4% for DDLT ($P = 0.20$). The 1-, 3-, and 5-year RFS rates according to the Hangzhou criteria were 94.0%, 77.3%, and 77.3% for LDLT *vs* 87.9%, 67.3%, and 65.3% for DDLT ($P = 0.20$). The 1-, 3-, and 5-year OS rates according to the Milan criteria were 86.1%, 68.8%, and 68.8% after LDLT *vs* 80.5%, 62.2%, and 59.8% after DDLT ($P = 0.53$). The 1-, 3-, and 5-year OS rates according to the UCSF criteria were 85.1%, 70.9%, and 70.9% for LDLT *vs* 75.8%, 59.2%, and 57.1% for DDLT ($P = 0.25$). The 1-, 3-, and 5-year OS rates according to the Hangzhou criteria were 86.3%, 69.6%, and 69.6% after
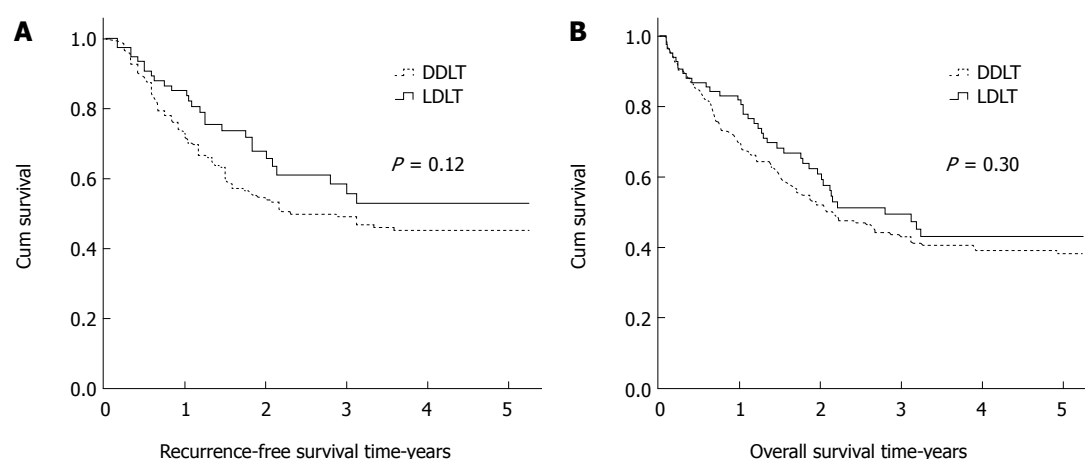
Figure 1 Recurrence-free survival (A) and overall survival (B) of hepatitis B virus-related hepatocellular carcinoma patients after living donor liver transplantation and deceased donor liver transplantation. LDLT: Living donor liver transplantation; DDLT: Deceased donor liver transplantation.
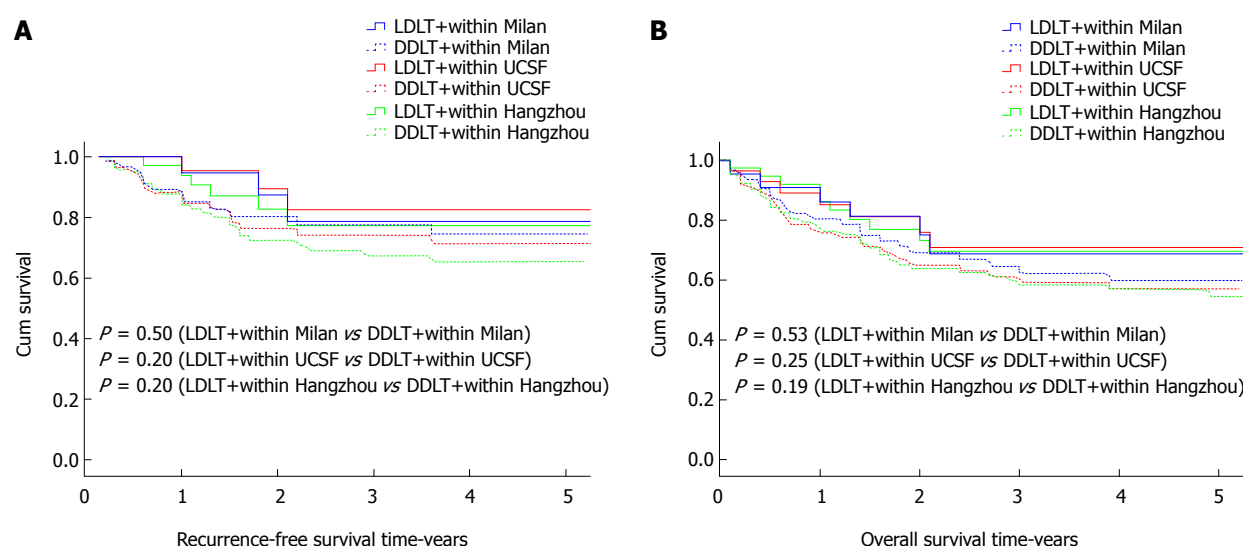
Figure 2 Recurrence-free survival (A) and overall survival (B) of hepatitis B virus-related hepatocellular carcinoma patients divided according to different criteria after living donor liver transplantation and deceased donor liver transplantation. LDLT: Living donor liver transplantation; DDLT: Deceased donor liver transplantation; UCSF: University of California, San Francisco.

LDLT *vs* 76.2%, 58.3%, and 56.6% after DDLT (*P* = 0.19). There were no significant differences in the RFS and OS rates between LDLT and DDLT patients divided according to the Milan, UCSF, and Hangzhou criteria (Figure 2).

DISCUSSION

Compared with DDLT, LDLT can shorten the pre-transplant waiting time and can also solve the issue of limited donors. LDLT is widely accepted as a treatment for patients with end-stage liver disease. At the same time, LT is the best choice for HCC because it can completely clear a tumor in the liver and solve the problem of liver cirrhosis. However, LT is greatly limited by the shortage of deceased donors. Since the first successful LDLT was performed in Australia in 1989, the shortage of donors had been partially resolved[8]. LDLT is currently considered an alternative for benign end-stage liver disease and liver malignancies. At our LT center, the frequency of LDLT is increasing. As of December 2012, 109 liver cancer patients had received LDLT at our center. However, in the early stages, the prognosis for HCC patients after LT was not satisfactory at our center due to the lack of unified criteria in mainland China. As a result, some advanced HCC patients underwent LT in those days. Some investigators in China have proposed some standards of HCC for LT, such as the Chengdu criteria (total tumor diameter ≤ 9 cm, no macro-vascular invasion, and no lymph node or extra-hepatic organ metastases) and the Hangzhou criteria (total tumor diameter ≤ 8 cm, or for total tumor diameter > 8 cm, histopathologic grade I or II and a preoperative AFP level ≤ 400 ng/mL)[26,27].

The outcomes of LDLT and DDLT for HCC are controversial. Several researchers have shown that, for HCC, the outcomes of LDLT are poorer than DDLT[19,22].

Some studies have suggested that the release of hepatotropic cytokines and the increased vascular inflow associated with hepatic regeneration may stimulate the growth of residual HCC cells, which has been the case in both animal and clinical human studies[28,29]. However, our results show that the RFS rate of LDLT for HCC is not significantly different compared to DDLT. Some authors have reported that poor outcomes may be due to the shorter waiting time and the surgical procedure for LDLT. Compared to DDLT, the waiting time for LDLT is shorter[19-21], with the latter often being referred to as "fast-track" transplantation due to this advantage[30]. Some investigators have indicated that at least 20%-30% of long-waiting candidates drop out before receiving transplantation because of tumor progression[30,31]. However, if the waiting time is short, doctors might not have adequate time to assess the biological behavior of the tumor. Thus, more patients with potentially aggressive tumors may have been selected to receive LDLT[11]. Moreover, some authors have suggested that the preoperative treatments in LDLT are not radical[18]. Our results show that there is a statistically significant difference in the preoperative adjuvant therapy between LDLT and DDLT, and that more patients received preoperative adjuvant therapy in the DDLT group. LDLT needs to preserve more of the inferior vena cava. Meanwhile, the longer hepatic artery and bile duct of the recipients should be reserved. The shorter waiting time and all of these surgical procedures may lead to the recurrence of HCC after LDLT[18].

Although LDLT is controversial for HCC, our experience indicated that there were no significant differences for either RFS or OS rates in HCC patients who underwent LDLT or DDLT at our LT center (Figure 2). The RFS and OS rates of HCC patients according to the Milan criteria were not significantly different after LDLT, while the RFS and OS rates of HCC patients according to the UCSF criteria were not significantly difference after LDLT and DDLT. We also demonstrated that the outcomes of HCC according to the Hangzhou criteria were not significantly different. Even the mean RFS and OS times for LDLT according to the different criteria were longer compared to the times for DDLT. Sandhu *et al*[16] reported that the type of transplant did not affect the HCC outcome. The authors demonstrated that, for HCC, RFS and long-time survival after LDLT or DDLT were similar. A study by Liang *et al*[15] also suggested that LDLT guarantees the same prognosis as DDLT for HCC according to the Milan criteria. Furthermore, Di Sandro *et al*[14] reported that LDLT guarantees the same long-term results as DDLT when the selection criteria for the candidates are the same. Although LDLT has a higher cost for the donor and the operative procedures are more complex, the overall financial burden is similar to DDLT. At the same time, LDLT can resolve issues involving allocation and the shortage of deceased donors[32-34]. Previous studies both at our own and other LT centers have demonstrated that LDLT has more advantages than DDLT, such as shorter waiting time, signifi-

cantly shorter cold ischemia time, and almost no warm ischemia injury[35,36]. With these advantages, LDLT ensures that more end-stage liver disease can receive optimal and timely therapy. At our center, the "no-touch" technique, use of a 5-Fu lavage in the recipients' peritoneal cavity, application of low-dosage immunosuppressive drugs, withdrawal of corticosteroids in the early stages, and control of HBV may play important roles in inhibiting the growth of HCC cells.

In conclusion, the results of our study demonstrate that LDLT does not increase the tumor recurrence of HCC compared to DDLT. The RFS and long-time survival times of LDLT for HCC are higher when compared to the times for DDLT. LDLT should be widely adopted in patients with benign end-stage liver disease and malignancies according to the Milan, UCSF, and Hangzhou criteria.

COMMENTS

Background
In the field of liver transplantation (LT), organ shortage is an urgent problem that needs to be resolved. Living donor liver transplantation (LDLT) plays an important role in combating the shortage of donated organs. However, some researchers demonstrated that tumor recurrence of LDLT was higher than deceased donor liver transplantation (DDLT) for HCC.

Research frontiers
The long-term outcomes of LDLT and DDLT for HCC are still a central issue of debate. This study included a large sample of HCC patients who received LT, of which 84 underwent LDLT. The results suggested that the recurrence-free survival and overall survival of LDLT and DDLT were similar.

Innovations and breakthroughs
These results suggest that LDLT does not increase the tumor recurrence of HCC compared to DDLT. The RFS and long-time survival times of LDLT for HCC are higher than those of DDLT.

Applications
With the current shortage of donors, LDLT should be widely adopted in patients with benign end-stage liver disease and malignancies according to the Milan, UCSF, and Hangzhou criteria.

Terminology
LT is the replacement of a diseased liver with some or all of a healthy liver from another individual. LDLT: a piece of healthy liver is surgically removed from a living individual and transplanted into a recipient. DDLT: a piece or a whole liver is transplanted from an individual with brain or cardiac death into a recipient.

Peer review
This current study compared the differences of RFS and overall survival of hepatitis B virus-positive HCC after living donor liver transplantation and deceased donor liver transplantation. Their results suggested that LDLT does not increase the tumor recurrence of HCC compared to DDLT. The data collection was detailed, and the analysis and results were full and accurate. The conclusion may provide some cross-references for other surgeon and investigators.

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RETROSPECTIVE STUDY

Chemotherapy for transarterial chemoembolization in patients with unresectable hepatocellular carcinoma

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Abstract

AIM: To compare the efficacy of different chemotherapeutic agents during conventional transarterial chemoembolization (cTACE) in the treatment of unresectable hepatocellular carcinoma (HCC).

METHODS: A retrospective review was undertaken of patients with unresectable HCC undergoing cTACE from May 2003 to November 2011. A total of 107 patients were treated with at least one cTACE session. Irinotecan (CPT-11) was used as a chemotherapeutic agent in 24 patients, gemcitabine (GEM) in 24 and doxorubicin in 59.

RESULTS: The time to progression and overall survival rates were significantly superior in patients treated with CPT-11 compared with the GEM or doxorubicin treated groups (11.4, 8.2, 9.5 mo, $P = 0.02$ and 21.7, 12.7, 14.5 mo, $P = 0.004$, respectively). Subgroup analysis showed that for intermediate-stage HCC, CPT-11 resulted in a significantly longer time to progression and overall survival compared with the GEM or doxorubicin treated groups ($P = 0.022$; $P = 0.003$, respectively). There were no significant differences in adverse events among the three groups ($P > 0.05$).

CONCLUSION: For patients treated with cTACE, the chemotherapeutic agent CPT-11 was significantly associated with improved overall survival and delayed tumor progression compared with GEM or doxorubicin. There were no significant differences in clinical adverse events between the three agents. CPT-11 thus appears to be a promising agent when combined with cTACE for the treatment of HCC.

Core tip: In the present study, we aimed to compare the efficacy of different chemotherapeutic agents during conventional transarterial chemoembolization (cTACE) in the treatment of unresectable hepatocellular carcinoma. Our study indicated that for patients treated with cTACE, the chemotherapeutic agent irinotecan (CPT-11) was significantly associated with improved overall survival and longer time to progression compared with gemcitabine or doxorubicin. There were no significant differences in clinical adverse events between the three agents. CPT-11 thus appears to be a promising agent when combined with cTACE for the treatment of hepatocellular carcinoma.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. The annual incidence ranges from < 10 cases per 100000 persons in North America and Western Europe to 50-150 cases per 100000 persons in parts of Africa and Asia, where HCC is responsible for a large proportion of cancer-related deaths[1,2]. The Barcelona Clinic Liver Cancer (BCLC) staging system directs therapy according to tumor stage, liver function status, physical status and cancer-related symptoms[3]. However, over 60% to 70% of patients with HCC are diagnosed at a late stage and therefore curative therapies such as resection, liver transplantation or local ablation therapy are not appropriate[4]. Transarterial chemoembolization (TACE) is the primary treatment used most frequently for unresectable HCC. TACE has been shown to improve survival when compared with best supportive care for unresectable HCC[5,6]. The rationale for using TACE is that intra-arterial chemotherapy using lipiodol and chemotherapeutic agents followed by selective vascular embolization will result in a strong cytotoxic effect combined with ischemia (conventional TACE or cTACE)[7,8].

However, there is a lack of data to support the use of one chemotherapeutic agent or combination of agents over another. Doxorubicin as a single agent is the most common chemotherapeutic agent used worldwide. In the United States, combination therapy is more often used, typically consisting of doxorubicin, mitomycin C and cisplatin. An adenosine triphosphate tumor chemosensitive assay system is a new promising regime as a single chemotherapeutic treatment for HCC. Cells of HCC are highly sensitive to various chemotherapy drugs: taxol 46%, CPT-11 (irinotecan) 44%, gemcitabine (GEM) 36%, mitomycin 14%, adriamycin 12%, cisplatin 8%, 5-fluorouracil oxalate (5-FU) 4%[9]; the higher the percentage, the higher the sensitivity. Thus, it is indicated that CPT-11 might be a potential drug for the treatment of HCC and prolong survival time of HCC patients.

CPT-11, a drug used for the treatment of cancer, prevents DNA unwinding by inhibition of topoisomerase 1. It is a semi-synthetic analogue of the natural alkaloid camptothecin and is activated by hydrolysis to SN-38, an inhibitor of topoisomerase 1. Inactivation follows by uridine diphosphate glucoronosyltransferase 1A1 glucuronidation. The inhibition of topoisomerase 1 by the active metabolite SN-38 eventually leads to inhibition of both DNA replication and transcription. In 2007, Takeba et al[10] suggested that the antitumor effects of SN-38 might include the mechanism of the mitochondria-apoptotic pathway inducing p53 activation. This newly discovered mechanism of action of CPT-11 might be useful as a treatment for patients with HCC. Currently, there are limited data available regarding the use of chemotherapeutic agents administered via cTACE in patients with HCC. This study evaluated the efficacy, tumor response, clinical adverse events, time to progression and overall survival benefit of three chemotherapy agents: CPT-11, GEM and doxorubicin.

MATERIALS AND METHODS

This study was approved by the ethics committees of the Dalian Medical University (No. 2013.012). As a retrospective medical records study, consents were not obtained. The records and personal information of all patients were anonymized prior to analysis.

Study design

This retrospective analysis was conducted on 107 patients with HCC who were treated with TACE-based therapy from May 2003 to November 2011 at the Second Hospital of Dalian Medical University of China. There were 95 men and 12 women with a mean age of 57 years (± 11 years). Hepatitis B virus was present in 81 of the 107 patients. The primary tumor was verified in all patients either by biopsy and histopathology or according to EASL criteria[11]. Briefly, non-invasive diagnosis of HCC was verified if a nodule of more than 2 cm within existing liver cirrhosis appeared arterially hypervascularized and with an enhanced venous "wash-out" on one contrast-enhanced imaging modality, with an AFP level exceeding 400 ng/mL. In patients with AFP levels below 400 ng/mL, a tumor greater than 2 cm had to show the above-mentioned dynamics of the contrast agent in two different imaging modalities.

Data evaluation was performed retrospectively and data were reported according to the standards defined by the Society of Interventional Radiology[12]. The study was performed in accordance with guidelines of the local institutional review board. A computed tomography (CT) scan was performed before the first chemoembolization to assess tumor size, multifocality, vascular invasion, morphological signs of liver cirrhosis and the presence of ascites. Etiology of liver cirrhosis, laboratory results including bilirubin, albumin, liver enzymes, prothrombin time (as Quick value or INR), thrombocytes, AFP and Eastern Cooperative Oncology Group status were retrieved from patient records. Based on these data, all patients were rated according to Child-Pugh[13,14], The Model for End-stage Liver Disease (MELD)[15], Cancer of the Liver Italian Program (CLIP)[16] and the BCLC[17]. Survival data were based on patients' records from our institution and follow-up information from their families.

Chemotherapy regimen and dosage

CPT-11 and 5-Fu were used as chemotherapy agents in the CPT-11 group, GEM and 5-Fu in the GEM group, and doxorubicin and 5-Fu in the doxorubicin group. The doses were CPT-11 130-180 mg/m^2, GEM 1000 mg/m^2, doxorubicin 30-40 mg/m^2, 5-Fu 500-600 mg/m^2. Physical condition of patients was also considered in the determination of the final doses.

Chemoembolization procedure

Digital subtraction angiography (DSA, Multistar, Siemens,

Erlangen, Germany) was performed before TACE to show vascular anatomy of the liver and to identify arterial feeders of the tumor. TACE was performed by selective catheterization of the hepatic segmental arteries nourishing the lesions. A 3-F coaxial microcatheter (TurboTracker 18; Boston Scientific, Cork, Ireland) was utilized. A co-mixture of iodised oil (Lipiodol UltraFluid; Laboratories Guerbet, Aulnay-sous-Bois, France) and chemotherapeutic agent (CPT-11, GEM or doxorubicin) with gelatine sponge particles (Spongostan Standard; Johnson and Johnson Medical Limited, Gargrave, Skipton, United Kingdom) was injected until a complete blockage of the tumor feeding branch was demonstrated. The doses of anticancer agent and lipiodol and the pieces of gelatine sponge particles used for TACE were determined based on the tumor size and extension of the lesions.

TACE was considered to be technically successful when target lesions were fully embolized and a complete blockage of the tumor feeding branch was demonstrated in the absence of immediate technical complications requiring treatment interruption. Complications were defined according to the Society of Interventional Radiology guidelines[18].

Follow-up

After the TACE procedure, patients recovered with approximately 12 h of bed rest in hospital. During the first 6 h, a clinical examination (abdominal evaluation and measurements of pulse rate, arterial blood pressure and body temperature) was performed every two hours. All patients underwent routine laboratory tests (liver enzyme biochemistry, AFP, routine blood) to assess peri-procedural complications and impact on liver function 7 d later after TACE.

One month after each cTACE procedure, a CT scan was performed in order to evaluate the tumor radiological response and then in all cases with complete response, scans were performed every three months in order to monitor the appearance of recurrence. Tumor response was assessed at CT by two expert abdominal radiologists according to the amended RECIST criteria[19,20]. Complete response (CR) was defined as the disappearance of any intratumoral arterial enhancement in all target lesions. All the other radiological responses were considered non-complete (non-CR) and categorized as partial response (PR), progressive disease (PD) and stable disease (SD) according to mRECIST criteria.

Viable tumor was defined as contrast uptake in the arterial phase and wash-out in portal venous and/or late venous phases. Contrast enhancement was visually assessed in the majority of cases. However, in doubtful cases at CT, quantitative measurements were obtained by placing a region-of-interest in specific areas in all phase images, according to Kim et al[21]. Repeated cTACE cycles were performed "on demand" upon the demonstration of viable tumour (non-CR) or intrahepatic recurrences in patients of Child-Pugh A and B.

Study endpoints

The primary endpoint of our study was overall survival. Secondary endpoints were: (1) safety and liver toxicity; (2) tumor response at one month; and (3) time to local tumor recurrence (within target lesion) and intrahepatic tumor recurrence (new lesions).

Statistical analysis

Continuous variables were reported as median and range. Comparisons among groups were calculated using non-parametric tests (Mann-Whitney and Wilcoxon). Categorical variables were compared with the χ^2 test. Survival analysis was performed with Kaplan-Meier statistics for all the patients as well as for the different Child-Pugh, MELD, CLIP, and BCLC stages. Median survival and CI were calculated. Differences in survival between the groups were assessed for statistical significance with the log-rank test. SPSS-software (version 15.0, SPSS Inc., Chicago, United States) was used for data evaluation and statistical analysis. A two-sided P value of less than 0.05 was considered statistically significant.

RESULTS

Baseline patient characteristics are shown in Table 1. The primary tumor was verified histopathologically in 17/107 of patients. In 90 patients, HCC was diagnosed based on radiological imaging procedures and AFP levels according to EASL criteria. A total of 53 patients were AFP-positive with levels greater than 400 ng/mL. Cirrhosis of the liver was present in 62 patients (58%) and thrombosis of a portal vein branch was present in 33 patients (31%). The mean tumor maximal diameter was 7.8 ± 4.1 cm. A mean of 2.0 ± 2.0 selective chemoembolization sessions were performed in each patient and the total number for all patients was 264.

Treatment response

Treatment response was evaluated one month after the first TACE session. In the CPT-11 group, 4 (16.7%) and 16 (66.7%) patients showed a CR and PR respectively, two patients (8.3%) progressed and two (8.3%) had SD. In the GEM group, 3 (12.5%) and 16 (66.7%) patients showed a CR and PR respectively, 2 (8.3%) progressed and 3 (12.5%) had SD. In the doxorubicin group, 3 (12.5%) and 16 (66.7%) patients showed a CR and PR respectively, 2 (8.3%) progressed and 3 (12.5%) had SD. There was no significant difference in treatment responses among the three groups.

Time to progression

During follow-up, the median time to progression in the CPT-11, GEM and doxorubicin groups was 11.41, 8.25 and 9.46 mo respectively. The time to progression was significantly longer in the CPT-11 group than the other two groups ($P = 0.02$, Figure 1A). Furthermore, subgroup analysis according to BCLC stage showed that

Table 1 Baseline characteristics

| | Total | CPT-11 | GEM | DDP + 5-FU | *P* value | |
	(*n* = 107)	(*n* = 24)	(*n* = 24)	(*n* = 59)	OS	PFS
Mean age ± SD (yr)	57.0 ± 11.0	61.0 ± 8.7	57.5 ± 12.4	56.0 ± 11.4		
Sex (M:F)	95:12	22:2	24:0	49:10		
HBV						
Absent/Present	26/81	7/17	6/18	13/46	0.583	0.734
Cirrhosis of the liver						
Absent/Present	45/62	13/11	6/18	26/33	0.003	0.011
Tumor maximal diameter (cm)	7.8 ± 4.1	8.0 ± 3.9	7.7 ± 4.1	7.4 ± 4.2		
≤ 5/> 5	30/77	4/20	5/19	21/38	0.361	0.165
Pathological T						
T1/T2/T3/T4	8/32/50/17	3/8/10/3	0/9/10/5	5/15/30/9	0.070	0.052
Pathological Stage						
Ⅰ/Ⅱ/Ⅲ/Ⅳ	7/26/57/17	3/6/10/5	0/6/13/5	4/14/34/7	0.013	0.022
TACE Sessions						
≤ 2/> 2	73/34	8/16	18/6	47/12	0.001	0.009
Initial AFP (ng/dL)						
≤ 400/> 400	59/48	18/6	11/13	16/43	0.095	0.157
Number of Tumor Single/Multiple	57/50	12/12	13/11	32/27	0.017	0.039
Vascular invasion						
Absent/Present	74/33	18/6	15/9	41/18	0.014	0.090
Child-Pugh						
A/B	98/9	20/4	23/1	55/4	0.746	0.930
BCLC Stage						
A/B/C	15/59/33	2/16/6	1/14/9	12/29/18	0.005	0.006
CLIP Score						
≤ 2/> 2	77/20	20/4	18/6	39/20	0.013	0.013
MELD Score						
≤ 6/> 6	74/33	17/7	16/8	41/18	0.914	0.610
ALB (g/L)						
≤ 40/> 40	49/58	9/15	7/17	33/26	0.033	0.073
TB (μmol/L)						
≤ 17/> 17	55/52	14/10	12/12	29/30	0.440	0.808
AST (U/L)						
≤ 40/> 40	23/84	8/16	8/16	7/52	0.947	0.958
ALT (U/L)						
≤ 40/> 40	32/75	11/13	8/16	13/46	0.456	0.522
Lipiodol (mL)						
≤ 10/> 10	62/45	11/13	15/9	36/32	0.997	0.369

Exp(B) stands for relative risk (RR). M: Male; F: Female; MELD: Model for End-stage Liver Disease; BCLC: Barcelona Clinic Liver Cancer Group; CLIP: Cancer of the Liver Italian Program; ALB: Albumin; TACE: Transarterial chemoembolization; CPT-11: Chemotherapeutic agent irinotecan; GEM: Gemcitabine; 5-FU: 5-fluorouracil oxalate; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BCLC: Barcelona Clinic Liver Cancer; OS: Oxidative stress.

for intermediate-stage HCC, time to progression was significantly longer in the CPT-11 group compared with the GEM or the doxorubicin groups (*P* = 0.022, Figure 1B). Univariate analysis revealed eight prognostic factors affecting tumor progression were recognized: cirrhosis of the liver, BCLC stage, CLIP stage, pathological stage, number of tumors (single/multiple), TACE sessions (≤ 2/> 2), PS score and chemotherapy agent used. In multivariate analysis, pathological stage (*P* = 0.021) and PS score (*P* = 0.032) were significant independent factors for tumor progression (Table 2).

Overall survival

Overall survival was evaluated from the time of first TACE session to the endpoint of death or the last follow-up time (31st December, 2012). In patients who died, the cause of death was progression of liver disease (74.8%), rupture of esophageal varices (18.7%) and others (6.5%). There were no treatment related deaths. There

was a lower rate of death in the CPT-11 group compared with the GEM or doxorubicin group (*P* = 0.02) due to less tumor progression. The median overall survival times in the CPT-11, GEM and doxorubicin groups were 21.68, 12.72 and 14.46 mo respectively. The cumulative survival rates at 12 and 24 months were 87.5% and 45.8% in the CPT-11 group, 66.7% and 0% in the GEM group and 69.5% and 22.0% in the doxorubicin group (Figure 2A). The overall survival was significantly higher in the CPT-11 group compared with the GEM or doxorubicin groups (*P* = 0.004). Subgroup analysis showed that the difference between the three groups was also significant in patients with intermediate-stage HCC (*P* = 0.003, BCLC B stage, Figure 2B). Univariate analysis revealed eight prognostic factors affecting overall survival: cirrhosis of the liver, BCLC stage, CLIP stage, pathological stage, number of tumors (Single/Multiple), TACE sessions (≤ 2/> 2), ALB and chemotherapy agent used. In multivariate analysis, the chemotherapy agent used was

At risk

GPT-11	24	11	7	4	3	2
GEM	24	4				
Dox	59	20	10	5	2	2

At risk

GPT-11	16	7	5	4	3	2
GEM	14	2				
Dox	29	6	4	2		
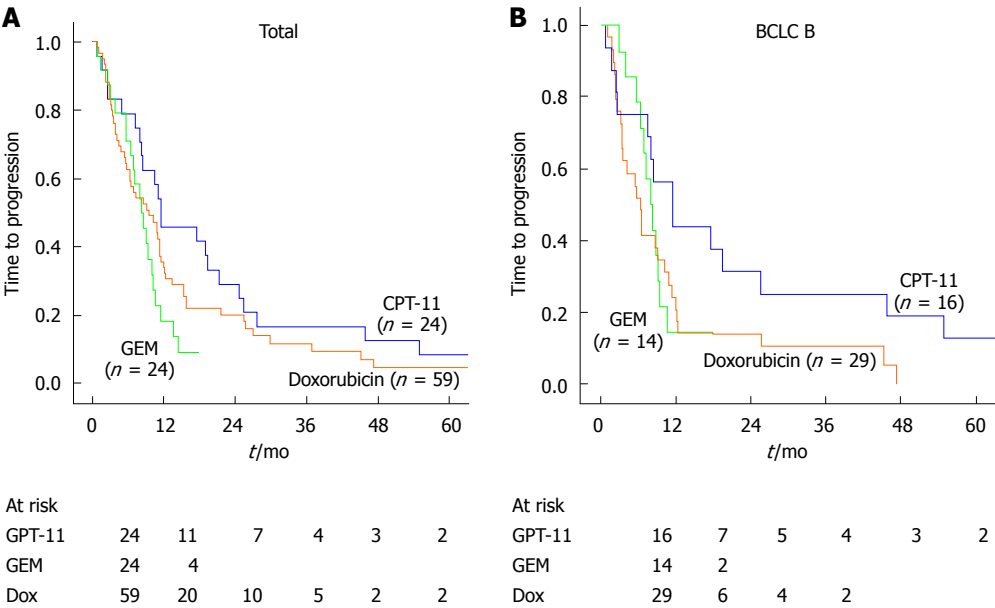
Figure 1 Time to progression in the chemotherapeutic agent irinotecan, gemcitabine and doxorubicin groups. A: There was a significant difference in time to progression among the three groups ($P = 0.018$). B: Time to progression in intermediate-stage HCC among the three groups ($P = 0.373$). HCC: Hepatocellular carcinoma; CPT-11: Chemotherapeutic agent irinotecan; GEM: Gemcitabine; BCLC: Barcelona Clinic Liver Cancer Group.

Table 2 Univariate and multivariate analysis for the factors that influence tumor progression

Factors	Univariate (P value)	Multivariate (P value)	Exp(B)	95%CI
Cirrhosis of the liver				
Absent/Present	0.025	0.928	1.070	0.242-4.729
Pathological stage				
I / II / III / IV	0.022	0.021	0.643	0.442-0.936
TACE Sessions				
≤ 2/> 2	0.009	0.083	1.272	0.969-1.670
BCLC Stage				
A/B/C	0.006	0.666	1.241	0.465-3.316
Number of Tumor				
Single/Multiple	0.039	0.734	0.839	0.305-2.309
CLIP Score				
≤ 2/> 2	0.013	0.466	0.822	0.485-1.392
Chemotherapy agent				
CPT-11/GEM/Doxorubicin	0.020	0.648	0.869	0.474-1.591
PS score				
1/2/2000	0.029	0.032	0.095	0.011-0.818

Exp(B) stands for relative risk (RR). TACE: Transarterial chemoembolization; BCLC: Barcelona Clinic Liver Cancer Group; CLIP: Cancer of the Liver Italian Program; CPT-11: Chemotherapeutic agent irinotecan; GEM: Gemcitabine.

a significant independent factor for overall survival ($P = 0.016$, Table 3). In addition, ALB ($P = 0.030$), pathological stage ($P = 0.012$) and number of TACE sessions ($P = 0.001$) were related to survival. These results suggest that the use of CPT-11 may be associated with a better prognosis in patients with HCC.

Treatment-related toxicity

Overall, adverse events were transient and tolerable and successfully managed with conservative treatment. Post-embolization symptoms, such as fever or pain, occurred in 23 patients and were reported as mild. There were no major complications or grade 4 liver toxicity[22] in either group within one week after cTACE. The most common adverse event was bone marrow suppression (37 patients) in the CPT-11, GEM and doxorubicin groups. Grade IV of bone marrow suppression was experienced in 1, 2 and 0 patients; 2, 3 and 4 patients had grade III; and mild elevation was seen in 3, 9 and 13 patients (grade I and II), respectively. Elevation of bilirubin was documented in three patients. Four patients experienced mild gastrointestinal symptoms (nausea or vomiting). Diarrhea occurred in only two patients treated with CPT-11 (Table 4).

DISCUSSION

Conventional transarterial chemoembolization is widely accepted as a predominantly palliative approach for
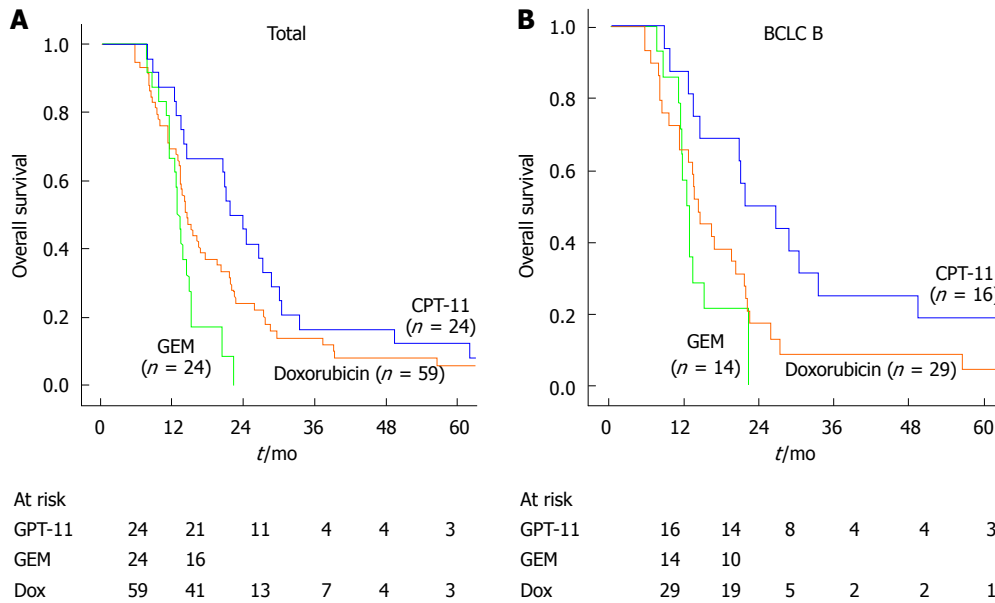
Figure 2 Overall survival rates in the chemotherapeutic agent irinotecan, gemcitabine and doxorubicin groups. A: Significantly better overall survival rates were observed in the chemotherapeutic agent irinotecan (CPT-11) group than in the GEM and doxorubicin group ($P = 0.004$). B: Overall survival rates in intermediate-stage HCC among the three groups ($P = 0.003$). GEM: Gemcitabine; HCC: Hepatocellular carcinoma.

Table 3 Univariate and multivariate analysis for the factors influencing survival rate

Factors	Univariate (*P* value)	Multivariate (*P* value)	Exp(B)	95%CI
Cirrhosis of the liver				
Absent/Present	0.003	0.083	5.114	0.806-32.436
Pathological Stage				
I / II / III / IV	0.013	0.012	0.485	0.276-0.851
TACE Sessions				
≤ 2/> 2	0.001	0.001	1.964	1.311-2.942
BCLC Stage				
A/B/C	0.005	0.061	0.183	0.031-1.078
Number of Tumor				
Single/Multiple	0.017	0.460	1.651	0.437-6.235
CLIP Score				
≤ 2/> 2	0.013	0.982	0.992	0.496-1.985
Chemotherapy agent				
CPT-11/GEM/Doxorubicin	0.004	0.019	0.407	0.192-0.863
ALB (g/L)				
≤ 40/> 40	0.033	0.030	0.834	0.709-0.982

TACE: Transarterial chemoembolization; BCLC: Barcelona Clinic Liver Cancer Group; CLIP: Cancer of the Liver Italian Program; GEM: Gemcitabine; CLIP: Cancer Liver Italian Program; ALB: Albumin; CPT-11: Chemotherapeutic agent irinotecan; GEM: Gemcitabine.

patients with HCC when surgical intervention is not appropriate. The rationale for TACE is that a powerful cytotoxic effect combined with ischemia followed by chemoembolization of the hepatic artery will result in therapeutic efficacy and survival benefit compared with supportive care[23]. If performed in a selective and sequential way, high concentrations of embolic and chemotherapeutic agents may offer effective local tumor control, whilst maintaining tolerable systemic concentrations reducing the risk of significant adverse events, such as liver failure and other clinical adverse events. This study demonstrated that local tumor control translates into long survival times for patients treated with more sessions of cTACE[23,24]. However, there is insufficient evidence of

chemotherapeutic agents used with cTACE to allow informed comparisons. Doxorubicin has been widely used as the chemotherapeutic agent of choice in cTACE, but with the development of new chemotherapeutic agents, such as CPT-11, GEM and oxaliplatin, comparative studies are needed to find the optimum agent for use in cTACE for the treatment of HCC.

This study is based on previous research on the application of the adenosine triphosphate tumor chemosensitive assay system as sole chemotherapy for HCC[9]. A comparison of CPT-11, GEM and doxorubicin agents used in cTACE for the treatment of HCC was performed. The time to progression and overall survival were significantly longer in patients treated with CPT-11.

Table 4 Treatment-related toxicity

	Grade I / II / III /IV		
	GEM	**CPT-11**	**Doxorubicin**
Aminotransferase elevation	2/4/2/0	4/2/0/0	7/2/1/0
Hyperbilirubinemia	1/0/0/0	1/0/0/0	2/0/0/0
Gastrointestinal toxicity	1/0/0/0	2/0/0/0	0/1/0/0
Post-embolization symptom	3/2/0/0	3/3/0/0	7/5/0/0
Bone marrow inhibition	4/5/3/2	2/1/2/1	8/5/4/0
Diarrhea	0/0/0/0	1/1/0/0	0/0/0/0

GEM: Gemcitabine; CPT-11: Chemotherapeutic agent irinotecan.

Additionally, liver toxicity or other clinical adverse events were not significantly different among the groups.

For tumor response, there was no significant difference among the groups. This may be explained by the hypothesis that embolization is more important than the chemotherapeutic agent used, but these agents may direct a powerful cytotoxic effect on hepatic cancer cells that determines time to progression and overall survival. Further research in this area is therefore warranted. Moreover, subgroup analysis according to the BCLC stage showed that for intermediate-stage HCC, the time to progression and overall survival were significantly better in the CPT-11 group compared with the GEM or doxorubicin groups ($P = 0.022$ and $P = 0.003$). As another new chemotherapy agent which may have potential, GEM in this study showed no advantages in cTACE with regard to the time to progression and overall survival compared with CPT-11 and even doxorubicin. From baseline characteristics in each groups, we found that the stage of patients in the CPT-11 and doxorubicin groups was relatively earlier than that in the GEM group, and more patients received extra gelatin sponge and microcatheter sessions in the CPT-11 and doxorubicin group than in the GEM group. These may be the reasons for the result produced in this study. However, we feel that our result is accurate, and more research should be performed to confirm it.

In this study, pathological stage was a prognostic factor in both the time to progression and overall survival. Earlier stage would be associated with a better prognosis in HCC patients, which is same as the conclusion in authoritative research and in guidelines of the National Comprehensive Cancer Network. Previous studies have shown that higher albumin (ALB) level is independently and significantly associated with improved survival duration[25-27]. From the multivariate analysis, patients with ALB > 40 g/L showed longer times of overall survival. However, due to the lack of ALB post-cTACE, the prognostic significance of ALB was not evaluated. This may be one potential point we can research. And we can see from this study that the patients who received more sessions (> 2) have a significantly different outcome compared with those who received only one or two sessions of cTACE regarding overall survival. This result is the same as reported in the study by Farinati *et al*[28]: the number of TACE courses and of embolizations is one

of the prognostic factors in HCC patients undergoing TACE. This indicates that cTACE is different from the curative treatments, and more sessions should be accepted by patients to control the time to progression. After progression has happened, more cTACE sessions should also be accepted to control the local tumor recurrence or new lesions in the liver, in order to prolong the time of overall survival. However, patients may omit cTACE sessions due to financial reasons, which affects the tumor response and overall survival.

This study has a number of strengths and limitations. Firstly, doxorubicin is widely used as the chemotherapeutic agent in cTACE, but there are few published studies assessing newer chemotherapy agents used with cTACE such as CPT-11 and GEM. Secondly, cTACE with CPT-11 showed improved time to progression and overall survival compared with GEM or doxorubicin. As for limitations, sample sizes of each group were not balanced, with a smaller number in the CPT-11 and GEM groups. Therefore, we can draw only preliminary conclusions regarding the potential value of CPT-11 in cTACE when compared with GEM and doxorubicin. Secondly, our study was a retrospective analysis with selection bias that may have influenced our findings. Further studies in a larger cohort are undoubtedly necessary to confirm these preliminary findings.

In the future, the combination of CPT-11-cTACE with drug-eluting beads or sorafenib is interesting with a view to performing more research. Sorafenib, a new multi-targeting drug, inhibits components of the Raf signaling pathway, VEGF, PDGF and RTKs, resulting in inhibition of tumor angiogenesis and proliferation. The efficacy and safety of sorafenib in the treatment of advanced HCC has been demonstrated in clinical practice[29] and in a phase III trial. Furthermore, it has been found to prolong survival times in patients with advanced HCC[30,31]. Studies are needed to compare the tumor response, time to progression and overall survival of patients treated with cTACE using sorafenib.

Conclusion

This study demonstrated that cTACE with CPT-11 could prolong the time to progression and overall survival in patients with HCC compared with GEM or doxorubicin. There were no significant differences in hepatic treatment-related toxicities and clinic adverse events. CPT-11 thus appears to be a feasible and promising choice of chemotherapy agent to use with cTACE for the treatment of HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. Over 60% to 70% of patients with HCC are diagnosed at a late stage and therefore curative therapies are not appropriate. Transarterial chemoembolization (TACE) is the primary treatment used most frequently for unresectable HCC. However, there is a lack of data to support the use of one chemotherapeutic agent or combination of agents over another. Chemotherapeutic agent irinotecan (CPT-11) (irinotecan), a drug used for the treatment of

cancer, prevents DNA unwinding by inhibition of topoisomerase 1. Many studies reported that CPT-11 might be a potential drug for the treatment of HCC and prolong survival time of HCC patients, but the effect has not been evaluated in TACE.

Research frontiers

Conventional transarterial chemoembolization (cTACE) is widely accepted as a predominantly palliative approach for patients with HCC when surgical intervention is not appropriate. The rationale for TACE is that a powerful cytotoxic effect combined with ischemia followed by chemoembolization of the hepatic artery will result in therapeutic efficacy and survival benefit compared with supportive care.

Innovations and breakthroughs

Doxorubicin has been widely used as the chemotherapeutic agent of choice in cTACE, but with the development of new chemotherapeutic agents, such as CPT-11, gemcitabine (GEM) and oxaliplatin, comparative studies are needed to find the optimum agent for use in cTACE for the treatment of HCC. Currently, there are limited data available regarding the use of chemotherapeutic agents administered *via* cTACE in patients with HCC. This study evaluated the efficacy, tumor response, clinical adverse events, time to progression and overall survival benefit of three chemotherapy agents: CPT-11, GEM and doxorubicin.

Applications

The study results suggest that the chemotherapeutic agent CPT-11 is significantly associated with improved overall survival and delayed tumor progression compared with GEM or doxorubicin. CPT-11 thus appears to be a promising agent when combined with cTACE for the treatment of HCC.

Terminology

CPT-11: a semi-synthetic analogue of the natural alkaloid camptothecin and activated by hydrolysis to SN-38, an inhibitor of topoisomerase-1. Inactivation follows by uridine diphosphate glucoronosyltransferase 1A1 glucuronidation. The inhibition of topoisomerase 1 by the active metabolite SN-38 eventually leads to inhibition of both DNA replication and transcription.

Peer review

The authors present the scope and limitations of the study and pointed out that the most important items are the small number of cases and the retrospective character of the study.

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CLINICAL TRIALS STUDY

Management of *Helicobacter pylori* infection in Latin America: A Delphi technique-based consensus

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Abstract

AIM: To optimize diagnosis and treatment guidelines for this geographic region, a panel of gastroenterologists, epidemiologists, and basic scientists carried out a structured evaluation of available literature.

METHODS: Relevant questions were distributed among the experts, who generated draft statements for consideration by the entire panel. A modified three-round Delphi technique method was used to reach consensus. Critical input was also obtained from representatives of the concerned medical community. The quality of the evidence and level of recommendation supporting each statement was graded according to United States Preventive Services Task Force criteria.

RESULTS: A group of ten experts was established. The survey included 15 open-ended questions that were distributed among the experts, who assessed the articles associated with each question. The levels of agreement achieved by the panel were 50% in the first round, 73.3% in the second round and 100% in the third round. Main consensus recommendations included: (1) when available, urea breath and stool antigen test (HpSA) should be used for non-invasive diagnosis; (2) detect and eradicate *Helicobacter pylori* (*H. pylori*) in all gastroscopy patients to decrease risk of peptic ulcer disease, prevent o retard progression in patients with preneoplastic lesions, and to prevent recurrence in patients treated for gastric cancer; (3) further investigate implementation issues and health outcomes of *H. pylori*

eradication for primary prevention of gastric cancer in high-risk populations; (4) prescribe standard 14-d triple therapy or sequential therapy for first-line treatment; (5) routinely assess eradication success post-treatment in clinical settings; and (6) select second- and third-line therapies according to antibiotic susceptibility testing.

CONCLUSION: These achievable steps toward better region-specific management can be expected to improve clinical health outcomes.

Core tip: By means of Delphi technique method, a multidisciplinary panel of Latin American experts releases a set of updated recommendations on diagnosis and treatment of *Helicobacter pylori* (*H. pylori*) infection for this region. Main recommendations include test and treat all symptomatic patients submitted to gastroscopy, use 14-d triple therapy or sequential therapy for first-line treatment, and to promote more information and demonstration projects to identify effective and safe strategies for control and prevention in areas with high prevalence of *H. pylori* infection and associated diseases.

INTRODUCTION

Latin America has a high burden of *Helicobacter pylori (H. pylori)* infection and associated diseases, particularly gastric cancer (GC). Clinical and public health management of this common bacterial infection needs to be adapted to different epidemiological situations. The last (and only) Latin-American Consensus Conference on *H. pylori* infection, published more than a decade ago, provided regional guidance for diagnosis and treatment[1]. Since then, important information has been gained on the role of *H. pylori* eradication in primary and secondary prevention of GC, availability of new diagnostic tests, decreasing efficacy of common antibiotic schemes and novel treatment approaches. A working group was convened to generate updated recommendations.

MATERIALS AND METHODS

Participants and evidence collection

Under the sponsorship of the Chilean Society of Gastroenterology (http://sociedadgastro.cl), the consensus organizing committee assembled a multidisciplinary group of adult and pediatric gastroenterologists, epidemiologists and basic scientists with expertise in various aspects of *H. pylori* infection and associated diseases, and evidence-based medicine. They were selected from a group of regional investigators particularly interested on *H. pylori* infection that had previously participated in a series of International Latin American Symposia on this topic. The organizing committee generated a list of questions relevant for Latin American countries related to diagnosis, long-term consequences and treatment of *H. pylori* infection. To address these questions, a member of the panel (RC) performed separate searches in PubMed® (United States National Library of Medicine, Bethesda, Maryland), retrieving reports published in English or Spanish up to May 2013. Search results were distributed and further supplemented as appropriate by individual panelists with data from regional databases (LILACS®, Latin America and the Caribbean Literature on Health Sciences, and SciELO®, Scientific Electronic Library Online), and abstracts presented at Latin American meetings. Each expert was required to answer one to three questions and to provide draft recommendation statements with rationales for consideration by all of the panelists. The quality of the evidence (Table 1) and the level of recommendation (Table 2) were graded following United States Preventive Services Task Force criteria[2,3].

Generation of the consensus

A modified three-round Delphi technique method[4] was used to reach consensus. Initial draft recommendation statements were compiled by the committee and distributed to the entire panel for the first assessment of agreement. A Likert-type scale (1, totally disagree; 2, disagree; 3, uncertain or with objections; 4, agree; and 5, totally agree) was used to measure agreement. In cases of disagreement or uncertainty (*i.e.*, score less than or equal to 3), panelists were required to submit comments and proposed changes. Recommendations were revised incorporating these opinions and returned to topic area experts for confirmation or reformulation. The updated statements were then judged by the entire panel as the second-round. In order to allow critical input from representatives of the concerned medical community, the recommendations were also presented to the roughly 400 gastroenterologists attending the XXXIX Chilean Congress of Gastroenterology and V International Symposium on *H. pylori* Infection in Viña del Mar, Chile, November 2012. The audience voted in real-time and provided additional oral comments. Final recommendations were revised as necessary to incorporate the public feedback, and translated from Spanish into English, for the third-round vote by the expert panel. Approved recommendations (*i.e.*, those with average score ≥ 4 on the Likert scale) are presented below.

RESULTS

What is the best use of noninvasive tests for the diagnosis of H. pylori infection?

Urea breath test: The consensus statement as follows: (1)

Table 1 Levels of evidence according to the study design[3]

Level of evidence	Description
Type I	Evidence obtained at least from one well-designed, randomized, controlled[1] trial or from a systematic review of randomized clinical studies
Type II	II-1 Evidence obtained from non-randomized, prospective, controlled[1] studies
	II-2 Evidence obtained from cohort observational studies[2] or case-control studies, preferably multi-centric
	II-3 Evidence obtained from case series
Type III	Opinion of authorities on the subject matter based on expertise, expert committees, case reports, patho-physiological studies or basic science studies

[1]A controlled study is a study where the intervention is managed by the researcher; [2]An observational study is a study where the intervention is not controlled by the researcher.

Table 2 Levels of recommendation according to the available evidence[3]

Recommendation	Description
A	The Consensus strongly recommends the mentioned intervention or service. This recommendation is based on high quality evidence, with a benefit that significantly exceeds the risks
B	The Consensus recommends the regular clinical use of the mentioned intervention or service. This recommendation is based on moderate quality evidence, with a benefit that exceeds the risks
C	The Consensus does not make any positive or negative recommendation regarding the mentioned intervention or service. A categorical recommendation is not provided, because the evidence (of at least moderate quality) does not show a satisfactory risk/benefit relationship. The decision has to be made on a case-by-case basis
D	The Consensus makes a negative recommendation against the mentioned intervention or service. The recommendation is based on at least moderate quality evidence, not showing any benefit or where the risk or damage exceeds the benefits of the intervention
I	The Consensus concludes that the evidence is insufficient, due to low-quality studies, heterogeneous results or because the risk/benefit balance cannot be determined

the urea breath test with ^{13}C (^{13}C-UBT) is a good non-invasive diagnostic test for *H. pylori* infection in adults, with high accuracy and easy implementation. (Evidence level II-1, grade of recommendation B; Agreement 4.7 ± 0.5); (2) in patients with peptic ulcer disease, when rapid urease test or histology is negative, a ^{13}C-UBT can be used to assess the presence of *H. pylori*. (Evidence level II-2, grade of recommendation B; Agreement 4.7 ± 0.5); and (3) the ^{13}C-UBT is a good method to confirm *H. pylori* eradication after treatment, both in adults and children, especially in those older than 6 years old. (Evidence level II-2, grade of recommendation B; Agreement 4.7 ± 0.5).

The rationale is that invasive methods are generally accepted to provide superior sensitivity and specificity for diagnosis for *H. pylori* infection. For non-invasive diagnosis, the ^{13}C-UBT is well-suited in different clinical situations[5,6]. Extensive reviews have consistently shown sensitivity between 88%-95% and specificity between 95%-100% using invasive methods as gold standard[7,8]. Thus, UBT may be used as part of the test-and-treat strategy in adult patients with dyspepsia, and also in epidemiological studies. However, in patients with increased risk of GC, endoscopic diagnosis strategies should be preferred[5].

Partial gastrectomy hampers the diagnostic accuracy of ^{13}C-UBT, dropping the sensitivity to 77% (95%CI: 72%-82%) and specificity to 89% (95%CI: 85%-93%)[9]. In contrast, the ^{13}C-UBT performs well in patients with peptic ulcer bleeding, as suggested by a meta-analysis reporting a sensitivity of 93% (95%CI: 90%-95%) and specificity of 92% (95%CI: 87%-96%)[10] When direct endoscopic tests for *H. pylori* infection are negative in patients with ulcer bleeding, a ^{13}C-UBT would be a suitable alternative. However, in areas with a high prevalence of H. pylori infection and rather low availability of diagnostic tests, such as the Latin American region, empirical *H. pylori* eradication immediately after bleeding would be appropriate and perhaps more cost-effective[11].

In children with dyspepsia and/or abdominal pain, the test-and-treat strategy has not been validated[12] and diagnosis of *H. pylori* infection is usually made by endoscopic methods. Moreover, performing UBT is relatively difficult in young children and its diagnostic accuracy is variable under 6 years old[13]. In children < 2 years of age, the ^{13}C-UBT may have false-positive results, requiring adjustments of the cutoff, pretest meal and urea dose. However, a recent meta-analysis[14] showed good diagnostic accuracy of ^{13}C-UBT in pediatric patients (sensitivity 96%, specificity 96%), especially in children > 6 years (sensitivity 97%, specificity 98%), but also in children ≤ 6 years (sensitivity 95%, specificity 94%).

There is extensive evidence from several high quality studies that ^{13}C-UBT is an excellent method to confirm eradication of *H. pylori* after antibiotic treatment in both children[10,13] and adults[15], despite the variability in the dose of marker, type of food, fasting period, type of analysis and cutoff point.

***H. pylori* HpSA:** The consensus statement as follows: (1) the monoclonal HpSA is an alternative for non-invasive diagnosis of *H. pylori* infection in adults and children, either pre- or post-eradication (Evidence level II-2, grade of recommendation B; Agreement 4.6 ± 0.7); and (2) in patients with peptic ulcer bleeding, the polyclonal HpSA could be considered for diagnosis of *H. pylori* infection after a negative direct test (Evidence level II-2, grade of recommendation C; Agreement 4.6 ± 0.7).

The rationale is that a meta-analysis of 22 observational studies comprising 2499 patients evaluated the accuracy of monoclonal HpSA for the initial diagnosis

of *H. pylori*[16]. Sensitivity was 94% (95%CI: 93%-95%), specificity 97% (95%CI: 96%-98%), positive likelihood ratio (LR+) 24 (95%CI: 15%-41%) and negative likelihood ratio (LR-) 0.07 (95%CI: 0.04-0.12) as compared to at least one independent diagnostic method[16]. Performance was superior with monoclonal than with polyclonal antigen tests (sensitivity 95% *vs* 83%, respectively).

Twelve studies evaluated the performance of HpSA after *H. pylori* eradication. A pooled analysis of those studies showed sensitivity of 93% (95%CI: 89-96%), specificity of 96% (95%CI: 94-97%), LR+ of 17 (95%CI: 12-23) and LR- of 0.1 (95%CI: 0.07-0.15). Again, sensitivity with monoclonal test was superior than with polyclonal test (91% *vs* 76%, respectively). Subgroup analyses, considering different gold standards study populations or study quality showed no significant differences in results[16].

Another meta-analysis, including 6 studies and 377 adult patients, evaluated the accuracy of HpSA in patients with upper gastrointestinal (GI) bleeding[10]. Only polyclonal tests were analyzed. Sensitivity was 87% (95%CI: 82%-91%), specificity 70% (95%CI: 62%-78%), LR+ 2.3 (95%CI: 1.4-4) and LR- 0.2 (95%CI: 0.13-0.3), with high between-study heterogeneity. No subgroup analyses were performed.

A meta-analysis of HpSA in children included 48 case-control studies with 5799 patients. Monoclonal ELISA tests (6 studies, 445 patients) showed the best performance, with sensitivity and specificity both 97%, LR+ 29.9, and LR- 0.03. Polyclonal ELISA tests (29 studies, 2460 patients) had sensitivity of 92%, specificity of 93%, LR+ of 16.2, and LR- of 0.09, with high heterogeneity ($P < 0.0001$)[17].

Serological tests: The consensus statement as follows: (1) serological tests are not recommended for clinical diagnosis of *H. pylori* in adults; either pre- or post-eradication (Evidence level II-3, grade of recommendation D; Agreement 4.5 ± 0.8). Western-blot might be considered as an alternative for non-invasive diagnosis of *H. pylori* infection in children (Evidence level II-2, grade of recommendation C; Agreement 4.5 ± 0.8); and (2) in areas with high risk of GC, serological tests are cost-effective for identification of asymptomatic *H. pylori*-infected individuals (Evidence level III, grade of recommendation C; Agreement 4.5 ± 0.8).

The rationale is that a number of different techniques exist for detection of antibodies against *H. pylori*, including solid phase assays (mostly in the ELISA format), agglutination tests (antigen binds to latex beads or gelatin), western blotting (useful for detection of response to different antigens) and immunochromatography tests. Performance of the different tests may vary in same population, and the same test will vary when tested on different populations[18]. Rahman *et al*[19] evaluated different kits in 82 patients from India. Current infection marker immunoblot showed the best accuracy, with sensitivity of 98% (95%CI: 91%-99%) and specificity of

90% (95%CI: 70%-99%). A study in 337 asymptomatic volunteers in China with the Assure® rapid test showed a sensitivity of 93% (95%CI: 89%-96%) and specificity of 91% (95%CI: 83%-95%), and six month after treatment the sensitivity was 86% and specificity 97%[18].

A meta-analysis of serological tests in children included 58 studies and 8336 patients. The ELISA-IgG tests (42 studies, 5632 patients) showed sensitivity of 79% (95%CI: 77%-81%), specificity of 92% (95%CI: 92%-93%), LR+ of 10.2 (95%CI: 8.1-13) and LR- of 0.19 (95%CI: 0.15-0.25), while IgA tests showed a sensitivity of only 43% (95%CI: 36%-49%). Western-blot tests (10 studies, 1119 patients) showed sensitivity of 91% (95%CI: 89%-93%), specificity of 89% (95%CI: 86%-92%), LR+ of 8.2 (95%CI: 5.1-13.3) and LR- of 0.06 (95%CI: 0.02-0.16). There was evidence of considerable heterogeneity[20].

Screening for *H. pylori* has been proposed as a cost-effective strategy in prevention of GC in high-risk populations[21]. A number of screening strategies are currently available but it is unknown which approach is the best. Using a Markov model, a serologic testing was more cost-effective than the ^{13}C-UBT in prevention of GC in Singapore Chinese males[22].

Is it necessary to seek and eradicate H. pylori infection in all patients undergoing upper GI endoscopy?

Consensus statement: Testing and eradication of *H. pylori* infection in all symptomatic patients undergoing upper GI endoscopy decreases the risk of peptic ulcer disease and its complications and may improve functional dyspeptic symptoms, but does not modify the clinical course of gastroesophageal reflux disease (GERD) disease. (Evidence level I , grade of recommendation B; Agreement 4.1 ± 1.1).

Rationale: Chronic *H. pylori* infection is strongly associated with both benign and malignant outcomes[23,24]. Universal testing and eradication of *H. pylori* infection in patients undergoing upper GI endoscopy, regardless of endoscopic findings, should be considered from both clinical and epidemiological perspectives. Main indications for upper GI endoscopy include dyspeptic or reflux symptoms. *H. pylori* eradication is justified in dyspeptic patients with normal endoscopy. A meta-analysis of 21 randomized controlled trials (RCT) suggested that *H. pylori* eradication is better than placebo to improve symptoms in patients with functional dyspepsia, with a relative risk (RR) reduction of 10% (95%CI: 6%-14%) and a number needed to treat (NNT) of 14 (95%CI: 10-25)[25]. In patients with GERD, available evidence suggests that in most cases there is no clinically significant interaction between GERD and *H. pylori* infection[5]. Current proton pump inhibitors (PPI) are able to compensate for any increase in acid secretion that might occur after eradication of *H. pylori*. A RCT of 231 *H. pylori*-positive patients with GERD, on long-term PPI therapy showed that *H. pylori* eradication did not worsen GERD

or require increased omeprazole maintenance dose[26].

From an epidemiological perspective, the majority of patients with GERD may require long-term treatment with PPIs. It has been suggested that in the presence of *H. pylori* infection acid suppression may increase the risk of gastric atrophy[27]. Although this intriguing hypothesis has not been confirmed[28], some recent clinical guidelines still include long-term PPIs as an indication for *H. pylori* eradication[5,6].

What is the role of H. pylori eradication in primary and secondary prevention of GC? What is the most appropriate age to eventually implement this action?

Primary prevention: The consensus statement is that the potential benefit of eradicating *H. pylori* in primary prevention of GC is highly suggested. However, there is insufficient evidence to justify large-scale implementation in the general population. Further studies should be performed on high-risk populations in Latin America to confirm the expected benefit and to evaluate potential adverse effects. (Evidence level Ⅰ, grade of recommendation C; Agreement 4.5 ± 0.5).

The rationale is that the potential benefit of *H. pylori* eradication in primary prevention of GC has been evaluated as a secondary end-point in RCT of preneoplastic lesions, including individuals with and without gastric atrophy. The most recent meta-analysis of those studies suggests that *H. pylori* eradication significantly reduces the risk of GC[29]. We updated this meta-analysis by including more recent data from two trials[30,31], and excluding one of two reports that was based on the same sample[32,33]. The updated summary RR was 0.6 (95%CI: 0.4-0.9), with low heterogeneity among trials (*PQ* = 0.7, I^2 = 0%). Notably, the observed association was primarily driven by a single large study from China[31].

Some international consensus reports[5,6,34] consider a population intervention to "test and treat" for *H. pylori* an effective strategy for GC prevention in high-risk communities and some evidence supports the cost-effectiveness of *H. pylori* eradication for GC prevention at the population level[21].

There are no empirical data addressing the most appropriate age for interventions to eradicate *H. pylori* infection. The trials described above have generally targeted older individuals because of their greater prevalence of preneoplastic lesions and faster progression to more advanced histologies. Nevertheless, a model projecting the potential reduction in lifetime GC risk and associated costs in a high-risk region in China, found that eradication at age 20 years is more cost-effective as compared to ages 30 or 40. The model assumed that new infections and reinfection are rare in adulthood, even in developing countries[21].

Secondary prevention: The consensus statement is that the eradication of *H. pylori* infection is recommended as a routine measure to prevent recurrence in GC patients receiving either subtotal surgical gastrectomy or endo-

scopic resection. (Evidence level l, grade of recommendation A; Agreement 4.8 ± 0.5).

The rationale is that the potential benefit of *H. pylori* eradication in GC secondary prevention, defined as therapy in early stages of disease, has been mainly evaluated in patients with early GC who underwent subtotal surgical or endoscopic resection. Although observational studies have shown inconsistent results[35-37], an open-label, RCT of prophylactic eradication in 544 patients found an OR of 0.4 (95%CI: 0.2-0.8) for metachronous GC and a NNT of 19[38].

H. pylori infection and gastric premalignant lesions

What is the effect of *H. pylori* eradication on gastric premalignant lesions?: The consensus statement is that in patients with gastric premalignant lesions, the eradication of *H. pylori* infection halts the progression of chronic atrophic gastritis and probably that of intestinal metaplasia. Although the evidence is still limited, current data favors the eradication of *H. pylori* infection in these patients. (Evidence level I for CAG and Ⅱ-1 for IM, grade of recommendation B; Agreement 4.6 ± 0.5).

The rationale is that the effect of *H. pylori* eradication on the histologic improvement of premalignant lesions has not been fully elucidated and remains controversial. There are few RCT, usually with shorter follow-up than required to demonstrate effect[39], and most reports and meta-analyses are based on prospective cohort studies. A RCT in Colombia included 795 adults with premalignant lesions, randomized to *H. pylori* eradication and/or antioxidants[40]. After 12 years of follow-up, a composite histopathological score showed 15% more regression and 14% less progression in subjects who became *H. pylori* negative. The effect was more evident for subjects with CAG than with IM at baseline (total regression 66% *vs* 20%, respectively)[30]. Another long-term RCT from China including 3365 subjects, showed a significant reduction in the combined prevalence of CAG, IM, dysplasia and GC, after 5 years (OR = 0.8; 95%CI: 0.6-0.95) and 9 years of follow-up (OR = 0.6; 95%CI: 0.5-0.8)[41]. The most recent meta-analysis included 3 RCT and 8 observational studies, comprising 2,658 patients with CAG or MI, followed for 1 to 6.7 years. The summary mean difference on histological score before and after *H. pylori* eradication showed significant differences only for corpus CAG (*P* = 0.006), but not for antral CAG or IM in any anatomical site[42]. The inclusion of mainly observational studies and the short mean follow up period may have influenced these results. There are several impediments to the proper assessment of reversibility of gastric premalignant lesions[43]. Further well designed and properly executed studies are needed.

What is the most appropriate follow-up strategy for patients with premalignant conditions?: The consensus statement is that high-risk gastric premalignant conditions, such as severe or extensive CAG, IM or dysplasia, require periodic follow-up. Endoscopic examina-

tion is recommended every 2-3 years for patients with moderate to severe CAG or IM, annually for those with low-grade dysplasia, and every 3-6 mo for those with high-grade dysplasia and no focal lesion on endoscopy. (Evidence level III, grade of recommendation B; Agreement 4.3 ± 0.8).

The rationale is that eradication of *H. pylori* may reduce GC incidence even in subjects with premalignant conditions[29], albeit less clearly than in subjects without them[44]. There is evidence from observational studies that GC risk of the intestinal type increases significantly with the severity of lesions[45,46]. The existence of a 'point of no return' is a widely accepted concept, although its precise location in the carcinogenic continuum is still unknown. The more advanced the preneoplastic lesion, the more likely it is that development of GC cannot be halted. In subjects with severe or extensive CAG or IM further monitoring is necessary even after *H. pylori* eradication, but there are no prospective studies evaluating various monitoring schemes. Risk stratification of patients with premalignant lesions should be based on histological assessment. When endoscopy is appropriate, the Sydney biopsy sampling protocol should be applied because of its worldwide acceptance[47]. The OLGA (Operative Link on Gastritis Assessment) histological staging is a recent proposal that considers the severity and distribution of gastric atrophy to assess the individual likelihood of progression to GC[48]. There is preliminary evidence of its prognostic accuracy[49]. More recently, the OLGIM histological staging, using IM instead of CAG because of its better interobserver agreement, has been shown to be of similar value[50]. Prospective multicenter studies in different epidemiological contexts are needed to further validate this new reporting format.

The Maastricht IV Consensus Report 2012 recommends that regular follow-up should be considered at 2-3 year intervals in moderate to severe atrophy and 3-6 mo intervals where there is dysplasia[5]. MAPS European guidelines recommend *H. pylori* eradication and endoscopic follow-up every 3 years for extensive CAG (corpus and antrum), annually for low-grade dysplasia, and immediate follow-up and then every 6-12 mo for high-grade dysplasia, with consideration of endoscopic or surgical resection of focal visible lesions[51]. Prospective studies, that should include factors such as age and family history of gastric cancer, are needed to test and validate the correct timing of follow-up.

What is the effectiveness of current therapeutics schemes to eradicate H. pylori? Which scheme should be the first option in Latin America?

Short (7 d) *vs* long (10-14 d) standard triple therapy: The consensus statement is that standard triple therapy should be administered for 14 d and include high-dose PPI to achieve the best possible eradication rate (Evidence level I , grade of recommendation B; Agreement 4.5 ± 0.8).

The rationale is that seven to 14 d of triple therapy

(TT), including a PPI, clarithromycin and either amoxicillin or metronidazole, has been the standard eradication regimen for the last 10 to 15 years. Many studies have evaluated the optimal duration of treatment. A meta-analysis showed a benefit of 7%-9% in the cure rate when comparing 7 d *vs* 14 d, but no differences between 7 and 10 d, with a per protocol (PP) eradication rate of 90%[52]. A more recent meta-analysis, including 21 studies showed no benefit in extending therapy over 7 d, although 14 d of treatment showed a favorable trend for the eradication rate in regimens including amoxicillin[53]. Most included studies were of low quality. Another meta-analysis concluded that higher doses of the more potent second-generation PPIs -namely, 40 mg of esomeprazole or rabeprazole twice a day- may increase cure rates by 8%-12% in comparison with standard doses[54]. The effectiveness of TT has shown a clear downward trend over the last years and in most recent studies eradication rates are below the 90% PP or 80% intention-to-treat (ITT) generally regarded as acceptable[55-57]. Rising antibiotic resistance is the most important determinant of treatment failure[58]. In Turkey, a small RCT showed 93% rate of PP eradication in patients infected with clarithromycin-susceptible strains treated for 14 d, compared to 63% in those treated for 7 d (P = NS), while in patients with clarithromycin-resistant strains, eradication rates were unacceptably low either after 14 or 7 d (60% and 27%, respectively)[59]. In Pakistan, 110 subjects infected with clarithromycin-susceptible strains, were randomized to 7 or 14-d high-dose PPI triple therapy (lansoprazole 60 mg twice daily). The eradication rate was 100% with the 14-d regimen and 92.7% (with the 7-d regimen (P = NS)[60].

Sequential therapy *vs* standard triple therapy: The consensus statement is that standard TT for 14 d is comparable to sequential therapy (ST) as empiric therapy for *H. pylori* infection in diverse Latin American populations. Sequential therapy is probably a better first-line alternative regimen in areas with high prevalence of clarithromycin-resistant strains (Evidence level I , grade of recommendation B; Agreement 4.6 ± 0.7).

The rationale is that the sequential therapy (ST), first introduced in Italy, consists of a 5-d dual therapy with a PPI (standard dose, bid.) and amoxicillin (1 g, bid) followed by a 5-d triple therapy with a PPI, clarithromycin (500 mg, bid) and metronidazole or tinidazole (500 mg, bid)[61,62]. This regimen could be more effective in the setting of high clarithromycin resistance, although would fail in the presence of dual clarithromycin and metronidazole resistance[63]. Many current clinical guidelines include both TT and ST as first-line regimens to treat *H. pylori* infection[64,65], and it has been argued that standard TT should be abandoned when clarithromycin resistance is more than 15%-20%, because the ITT eradication rates are usually less than 80% in this setting[5,66]. Both regimens have been compared in many RCT. A meta-analysis by Jafri *et al*[67] comparing ST with TT (7 or 10

d), included 10 RCT and 2747 patients. Eradication rate was significantly higher for ST than TT (93.4% *vs* 76.9%, respectively; *P* < 0.05). A second meta-analysis by Gatta *et al*[68] comprising 3006 patients also favored ST. The OR for *H. pylori* eradication was 3.0 (95%CI: 2.5-3.6), giving a NNT of 6. In patients with clarithromycin-resistant strains, the OR was 10 (95%CI: 3.0-35), but the numbers studied are small. The latest published meta-analysis by Tong *et al*[69], including 11 RCT, demonstrated superiority of ST over 7-d or 10-d TT, with a RR of 1.2 (95%CI: 1.2-1.3), and 1.2 (95%CI: 1.1-1.2), respectively. Limitations of all of these meta-analyses are that most of the included studies were conducted in Italy, few patients had clarithromycin-resistant strains and 14-d TT was not used. Some RCTs conducted in Iran[70], India[71] and South Korea[72] have failed to demonstrate superiority of ST over 10 or 14-d TT. In Taiwan, 900 adults were randomized to either 14-d or 10-d ST, or 14-d TT. The eradication rate was 91%, 87% and 82%, respectively. Treatment efficacy was significantly better for the ST-14 compared to TT-14 regimen (NNT of 12 on ITT analysis; *P* = 0.003)[73]. Finally, a recent updated analysis added data from 10 recent RCT to the 3 previous meta-analyses, totaling more than 5000 patients. *H. pylori* infection was eradicated in 86% (95%CI: 84.7-87.3) of patients treated with ST and in 75.3% (95%CI: 73.8-76.9) of patients with TT (*P* < 0.001), corresponding to a NNT of 9. They concluded that comparison between ST and 14-d TT deserves further investigations[74].

There are also relevant studies in pediatric populations. A study from Belgium showed superiority of ST only in patients with clarithromycin and metronidazole susceptible strains[75]. In Poland, a RCT found higher eradication rates with ST over TT for 7 d, although with borderline significance[76]. A meta-analysis including a total of 857 children aged 3-18 years, showed eradication rates of 78% with ST and 71% with TT (RR = 1.14, 95%CI: 1.06-1.23; NNT = 15). ST was superior to 7-d TT, but was not significantly better than 10-d or 14-d TT[77].

Regarding treatment regimens, the most important Latin American study is a recent multicenter RCT comparing 14-d TT *vs* 5-d concomitant (lansoprazole, amoxicillin, clarithromycin and metronidazole) and 10-d ST in seven sites (Chile, Colombia, Costa Rica, Honduras, Nicaragua, and Mexico), including 1463 participants[78]. The ITT eradication rate with TT an ST was similar (82.2% and 76.5%, respectively, *P* = NS). An updated evaluation showed that the estimated eradication success rate after 1 year of follow-up was virtually the same for both TT and ST (80.4% and 79.8%, respectively)[79]. Because both regimens just border the acceptable efficacy limit (80% ITT), there is an important space for improvement, and more efficacious treatment schemes are clearly needed.

Levofloxacin-based triple therapy: The consensus statement as follows: (1) a quinolone-based regimen is a good alternative second-line therapy, especially when bismuth is not easily available (Evidence level I , grade of recommendation A; Agreement 4.6 ± 0.5); and (2) a quinolone-based regimen might be considered as a first-line alternative regimen in areas with high prevalence of clarithromycin-resistance and low quinolone resistance (Evidence level I , grade of recommendation C; Agreement 4.6 ± 0.5).

The rationale is that both levofloxacin-based triple therapy (LBTT) (PPI, levofloxacin and amoxicillin) and the classical bismuth-based quadruple therapy (BBQT) (PPI, bismuth, tetracycline and metronidazole) have been recommended as second-line therapy by the Maastricht IV Consensus Report[5] and other international clinical guidelines[80,81]. Two meta-analyses evaluated the efficacy of LBTT as second-line therapy, showing higher eradication rates compared to 7-d BBQT with an OR of 1.80 (95%CI: 0.94-3.46)[82] and less adverse events than BBQT[82,83]. A subsequent meta-analysis including 13 RCT showed that the eradication rates of the two regimens were similar (OR = 1.43; 95%CI: 0.82-2.51) except for subgroup analysis comparing 10-d LBTT with 7-d BBQT (OR = 4.79, 95%CI: 2.95-7.79, *P* < 0.00001)[84]. The more recent meta-analysis included 14 RCT comparing 7 or 10-d LBTT with 7-d BBQT. Both 7-d regimens showed comparable efficacy, with eradication rates of 70.6% and 67.4%, respectively, whereas the 10-d LBTT was significantly better than 7-d BBQT (eradication rate 88.7% *vs* 67.4%, *P* < 0.001). LBTT regimens were more effective in European than in Asian populations (78.3% *vs* 67.7%, *P* = 0.05)[85]. All meta-analyses showed that LBTT for 10 d is more effective than for 7 d and better tolerance for LBTT than for BBQT.

Levofloxacin-based therapies have also been studied as first-line therapy, with inconsistent results. A non-randomized Dutch study compared two 7-d LBTT, with either amoxicillin or clarithromycin. ITT eradication rates were 96% and 93%, respectively, probably reflecting a very low local resistance to quinolones[86]. A RCT from the Middle East compared the same two LBTT with 7-d standard TT. ITT eradication rates were of 84.7% and 90.6% for amoxicillin and clarithromycin LBTT respectively *vs* 78.6% with TT (*P* < 0.001)[87]. Another RCT from Spain compared LBTT with standard TT, both for 10 d. ITT cure rates were similar (75.0% *vs* 82.8%, *P* = NS), perhaps reflecting the increasing levofloxacin resistance rate in this region. A RCT from South Korea, including 300 patients, compared 7-d LBTT with 7-d standard TT and with a quadruple regimen including PPI, levofloxacin, amoxicillin and rifaximin. The ITT eradication rate was higher with TT than with LBTT (77.8% and 65.3%, respectively, *P* = 0.05) while the rifaximin-based quadruple regimen was not inferior to TT[88]. Levofloxacin-based sequential or quadruple regimens have also been tried as first-line options, with better results than standard TT[89,90]. Based on this large body of clinical trial data, LBTT shows similar or better outcomes compared with other current first-line therapies. Under

exceptional circumstances, such as populations with low quinolone resistance (< 10%) and high clarithromycin resistance (> 15%-20%), this combination might be considered as a first-line treatment option for patients with no previous quinolone exposure[64,91].

Concomitant quadruple therapy: The consensus statement is that concomitant quadruple therapy for 10 or 14 d should be studied in Latin America and may be a good first or second-line alternative in areas with high prevalence of dual resistance to clarithromycin and metronidazole (Evidence level Ⅱ-1, grade of recommendation C; Agreement 4.3 ± 0.5).

The rationale is that the so-called "concomitant therapy" is a non-bismuth-containing quadruple regimen, including a PPI (standard dose, bid), clarithromycin (500 mg, bid), amoxicillin (1 g, bid) and metronidazole or tinidazole (500 mg, bid) and was designed primarily to overcome antibiotic resistance to TT. It has been used for 3 to 14 d but direct comparisons between variable durations of treatment are lacking[63,92,93]. A meta-analysis including 5 RCTs and 576 subjects compared concomitant quadruple therapy (CQT) (3 to 5 d) with standard TT (5 to 10 d). Pooled estimates showed ITT eradication rate of 90.8% and 79% for CQT and TT, respectively. The OR was 2.86 (95%CI: 1.7-4.7)[94]. Another meta-analysis suggested that CQT may overcome resistance to either clarithromycin or metronidazole[95]. CQT is less complex than ST as this regimen does not involve changing drugs halfway through and may be assembled by adding metronidazole or tinidazole to standard TT. A head-to-head non-inferiority trial of 10-d ST and 10-d CQT showed that they were equivalent (ITT eradication rate of 92.3% and 93.0%, respectively)[84]. Dual resistance to clarithromycin and metronidazole did not influence the level of eradication in the CQT group, but significantly affected efficacy of ST, although the low number of patients precludes a clear conclusion[63].

A Turkish RCT compared a modified BBQT (PPI, bismuth, tetracycline and amoxicillin) with a modified CQT (PPI, tetracycline, amoxicillin and metronidazole), both for 10 d, as first-line therapy. The ITT eradication rates were similar and unsatisfactory (79% and 74%, respectively; *P* = NS) probably because of antibiotic resistance[96]. In a Spanish RCT, patients with clarithromycin-susceptible strains were randomized to receive TT *vs* CQT, while those with clarithromycin-resistant strains were randomized to ST *vs* CQT[97]. For clarithromycin-susceptible patients, CQT was significantly better than TT (ITT eradication rate 92% *vs* 70%, respectively; *P* = 0.02). For clarithromycin-resistant and dual-resistant strains (9 cases each), the eradication rates were non-significantly better with CQT. In the same study, 209 consecutive naive *H. pylori*-positive patients without susceptibility testing were empirically treated with 10-d CQT, with an ITT eradication rate of 87% (95%CI: 83%-92%).

In Latin America, the previously mentioned multi-center RCT comparing some recommended first-line empirical regimens, included one arm with 5-d CQT[78]. Although TT had appeared to be superior to ST and CQT at 6 to 8 wk, there were only modest and non-significant differences in 1-year outcomes among the 3 treatment groups[79].

What is the clinical usefulness of assessing the susceptibility of H. pylori to antibiotics?

Consensus statement: Determination of antibiotic susceptibility of *H. pylori* before treatment may improve the effectiveness of therapy and should be used when available, particularly in populations with high prevalence of resistance (Evidence level Ⅰ, grade of recommendation B; Agreement 4.0 ± 0.6).

Antibiotic resistance of *H. pylori* should be monitored by systematic surveillance in all countries throughout the region (Evidence level Ⅲ, grade of recommendation B; Agreement 4.0 ± 0.6).

After a treatment failure, the design of second and third line therapies should be based on *H. pylori* antibiotic susceptibility to the greatest extent possible (Evidence level Ⅱ-3, grade of recommendation B; Agreement 4.0 ± 0.6).

Rationale: Clarithromycin resistance is the most important factor in explaining the increasing failure of standard TT[95], and has been correlated with the consumption of clarithromycin in the general population[98].

In Latin America, there is no surveillance system of *H. pylori* antimicrobial susceptibility. A meta-analysis of observational studies evaluating *H. pylori* strains in Latin American populations found high frequencies of primary antibiotic resistance, including summary prevalences of 12% for clarithromycin, 53% for metronidazole, 4% for amoxicillin, 15% for fluoroquinolones, and 8% for dual clarithromycin and metronidazole[99]. It has been suggested that standard TT should be used only when resistance of *H. pylori* to clarithromycin is less than 15%-20%[5] or after susceptibility testing has confirmed clarithromycin sensitivity[95].

Some studies have compared the effectiveness of empiric therapy *vs* therapy guided by antibiotic susceptibility (tailored therapy). A meta-analysis, comprising 5 RCT and 701 patients, showed that tailored TT had a higher ITT-eradication rate than empiric TT (RR = 0.84; 95%CI: 0.77-0.90) and suggested that tailored therapy may be cost-effective[100]. Several methodological weaknesses may limit the validity and generalizability of this meta-analysis, including that 4 of the studies came from Italy and cost analysis is based in only one study[101].

Culture of *H. pylori* may be difficult, even in expert hands[102] and sensitivity values of 55%-73% have been reported in some trials[103-106]. Few Latin American microbiological laboratories routinely perform culture and susceptibility studies of *H. pylori*, and standardization of culture media, culturing methods, and interpretative values for susceptibility testing of isolated strains is lacking.

The increasing availability of PCR-based approaches (not requiring culture) to evaluate antibiotic susceptibility may facilitate the implementation of these techniques[107]. A systematic effort to monitor the regional frequency and evolution of *H. pylori* antibiotic resistance would be very helpful for designing the best options for empiric treatment.

After a treatment failure, culture and standard susceptibility testing of *H. pylori* has been recommended, while after a second failure it should be performed in all cases[5]. However, there is limited evidence to sustain these recommendations. A study including 94 consecutive patients with 2 previous failures found resistance to metronidazole in 100%, to clarithromycin in 95%, to levofloxacin in 31% and to tetracycline in 5% of cases. Patients were treated with a culture-guided, third-line regimen, most with a 7-d BBQT including omeprazole, bismuth, doxycycline and amoxicillin. ITT eradication rate was 90%[108]. Another open prospective, multicenter study included 41 patients with 2 previous failures. Despite the use of two-week, high-dose, quadruple and culture-guided combinations of drugs, overall eradication rate was only 60%[109].

Recurrence of H. pylori infection after treatment

In which clinical situations eradication should be confirmed?: The consensus statement is that because of the declining efficacy of current therapies, *H. pylori* testing should be offered to all patients after eradication therapy, especially when persistent infection may be associated to clinically relevant disease risks, such as in patients with peptic ulcer disease, GC or mucosa-associated lymphoid tissue (MALT) lymphoma. (Evidence Level Ⅱ-1, grade of recommendation B; Agreement 4.3 ± 0.7).

The rationale is that because of the declining efficacy of current therapies, *H. pylori* testing should be offered to all patients after treatment, but is mandatory when the treatment failure may be associated to clinically relevant risks, such as in patients with complicated ulcer disease, with gastric MALT lymphoma or after endoscopic or surgical resection of GC[80,110-112]. However, cost-effectiveness of this strategy has not been determined. Testing should be done at least 4 wk after treatment, although proposals have been made to extend this period to 6 or 8 wk.

Peptic ulcer rebleeding virtually does not occur after *H. pylori* eradication[113,114], and bleeding recurrence is related to persistent or recurrent infection or concurrent NSAIDs[115]. Because persistent *H. pylori* infection poses a risk of a potentially serious complication, a second-line therapy is mandatory in this situation.

H. pylori play a causative role in the development of gastric MALT lymphoma and the eradication of *H. pylori* leads to a complete remission in 50%-90% of cases[80]. In a systematic review, data from 32 studies and 1,408 patients with gastric MALT lymphoma treated only by *H. pylori* eradication, demonstrated a remission rate of 77.5% and a relapse rate of 7.2% after 10 to 75 mo of follow up. Only 17% of relapses were related to recurrence of *H. pylori*, but lymphoma was cured by additional eradication therapy in all these patients[116].

How to define reinfection? What is the reinfection rate in Latin America?: The consensus statement as follows: (1) recurrence of *H. pylori* infection after treatment is variable in Latin America, but considerably higher than in developed countries, probably due to a higher frequency of reinfection. (Evidence level Ⅰ; grade of recommendation B; Agreement 4.0 ± 0.4); and (2) good-quality information about long-term risk of reinfection is lacking and should be addressed in future studies (Evidence level Ⅲ, grade of recommendation C; Agreement 4.0 ± 0.4).

The rationale is that recurrent *H. pylori* infection following apparently successful eradication can be due to a recrudescence (defined as infection by the same strain) or reinfection (*i.e.*, infection with a new strain). Because culture of *H. pylori* is uncommon in clinical practice, reinfection has been conventionally defined as the situation where tests for *H. pylori* infection, which were negative for 12 mo after eradication treatment, later become positive[117]. *H. pylori* recurrence within the first year after eradication seems likely to represent a mixture of recrudescence and reinfection, the former predominant[118], whereas reinfection dominates in subsequent years, and the overall annual risk of recurrence tends to diminish[119]. Recurrence risk is generally directly proportional to the frequency of infection in the population[120,121] and inversely proportional to the efficacy of the initial treatment[122]. In a review of more than 100 studies, the annual recurrence risk ranged from 3.4% (95%CI: 3.1%-3.7%), in high-income countries, to 8.7% (95%CI: 8.8%-9.6%) in lower-income countries[123]. In a meta-analysis of 17 studies, comprising more than 5000 patients followed for at least one year, the annual recurrence rates were 2.7% and 13% for developed and developing countries, respectively. The recurrence during the first year was similar, while nested meta-analysis of cases with a negative 12-mo UBT and a longer follow-up revealed an annual recurrence rate of 1.45% in developed countries and 12% in developing countries[15]. Only one of the studies came from Latin America[118]. Latin American studies with at least 50 person-years of follow-up showed 1-year recurrence risk from 0% to 17.3%[30,118,119,124,125]. A recent study evaluating the risk of recurrent *H. pylori* infection 1 year after successful therapy in 1091 subjects from 7 different Latin American communities found a recurrence risk of 11.5% (95%CI: 9.6%-13.5%). The recurrence rate significantly differed according to the study site, ranging from 6.8% in Costa Rica to 18.1% in Colombia (*P* = 0.03). Predictors of failed eradication were having more children in the household and poor adherence to initial therapy[79], suggesting that both recrudescence and reinfection are components of 1-year recurrence in this study. There is little information regarding the long-term recurrence rate of *H. pylori* infection in Latin America. A Brazilian study of 115 patients followed during 2 to 5 years showed an annual reinfection rate of 1.8%[124]. A

Chilean study of 96 patients followed for a mean of 37.2 mo showed a reinfection rate of 1.5% during the second and third year after treatment[119].

DISCUSSION

The high burden of *H. pylori* associated diseases in Latin America demands the attention of the public health community. The consensus statements presented here represent locally adapted strategies to control this serious problem.

However, some limitations should be considered. First, there is a shortage of locally-generated evidence about some of the selected topics. Second, it is important to note that factors such as accessibility of UBT, HpSA and antibiotic susceptibility testing, affordability, and differences in clinical setting between rural and urban areas of Latin America, not addressed in our study, may also influence the applicability of our recommendations.

Finally, our systematic review indicated that future epidemiological and clinical research should focus on (1) potential benefits and adverse effects of population-based eradication for primary prevention; (2) appropriate follow-up strategies for patients with advanced premalignant lesions; (3) identification of alternative and superior first-line therapies; (4) estimating long-term risk of reinfection; and (5) periodic and representative assessment of resistance to first- and second-line antibiotics. Better region-specific evidence is needed to inform future management toward improving clinical and population health outcomes.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection affects more than 80% of adult population across the Latin American region. Gastric cancer, an associated disease, is common and has a poor prognosis. The optimal management of this bacterial infection is evolving end needs to be adapted to local epidemiological situations. A multidisciplinary panel of Latin American experts conducted a structured analysis of the current literature on some relevant topics about *H. pylori* infection.

Research frontiers

Indications of *H. pylori* eradication for epidemiological reasons are still uncertain and the efficacy of common antibiotic schemes shows a progressive decline. More studies are needed, but in the meantime, consensual evidence-based recommendations can help to guide clinical practice.

Innovations and breakthroughs

Using the Delphi technique method to reach a consensus among experts after assessing the quality of the available evidence is a reasonable approach for generating up-to-date practical recommendations on *H. pylori* infection.

Applications

The statements of the current evidence-based clinical practice review provide a rationale for current management of *H. pylori* infection in clinical practice.

Terminology

The Delphi technique is a method for gathering data from specialists within their domain of expertise to achieve a convergence of opinion on a specific real-world issue. The Delphi technique is well suited as a method of consensus building in scenarios where evidence-based recommendations are insufficient or uncertain.

Peer review

To optimize diagnosis and treatment guidelines for *H. pylori* infection in Latin American countries, a group of gastroenterologists, epidemiologists and basic reviewed and discussed all relevant clinical data present in literature to arrive at recommendations for the clinical management of *H. pylori* infection. Fifteen key clinical questions were proposed and a modified Delphy method was used to reach consensus. The paper is well written and it is complete.

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OBSERVATIONAL STUDY

Age-related differences in response to peginterferon alfa-2a/ribavirin in patients with chronic hepatitis C infection

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Abstract

AIM: To evaluate the safety and efficacy of pegylated interferon alfa-2a and ribavirin therapy in elderly patients with chronic hepatitis C infection.

METHODS: Patients characteristics, treatment results and safety profiles of 4859 patients with hepatitis c virus (HCV) infection receiving treatment with pegylated interferon alfa-2a and ribavirin were retrieved from a large ongoing German multicentre non-interventional study. Recommended treatment duration was 24 wk for GT 2 and GT 3 infection and 48 wk for GT 1 and GT 4 infection. Patients were stratified according to age (< 60 years *vs* ≥ 60 years). Because of limited numbers of liver biopsies for further assessment of liver fibrosis APRI (aspartate aminotransferase - platelet ratio index) was performed using pre-treatment laboratory data.

RESULTS: Out of 4859 treated HCV patients 301 (6.2%) were ≥ 60 years. There were more women (55.8% *vs* 34.2%, *P* < 0.001) and predominantly GT 1 (81.4% *vs* 57.3%, *P* < 0.001) infected patients in the group of patients aged ≥ 60 years and they presented more frequently with metabolic (17.6% *vs* 4.5%, *P* < 0.001) and cardiovascular comorbidities (32.6% *vs* 6.7%, *P* < 0.001) and significant fibrosis and cirrhosis (F3/4 31.1% *vs* 14.0%, *P* = 0.0003). Frequency of dose reduction and treatment discontinuation were significantly higher in elderly patients (30.9% *vs* 13.7%, *P* < 0.001 and 47.8% *vs* 30.8%, *P* < 0.001). Main reason for treatment discontinuation was "virological non-response" (26.6% *vs* 13.6%). Sustained virological response (SVR) rates showed an age related difference in patients with genotype 1 (23.7% *vs* 43.7%, *P* < 0.001) but not in genotype 2/3 infections (57.7% *vs* 64.6%, *P* = 0.341). By multivariate analysis, age and stage of liver disease were independent factors of SVR.

CONCLUSION: Elderly HCV patients differ in clinical characteristics and treatment outcome from younger patients and demand special attention from their practitioner.

Key words: Hepatitis C virus infection; Older patients;

Patients ≥ 60 years; Geriatric; Therapy; Non-interventional study; Epidemiology

Core tip: There are concerns to initiate treatment in elderly patients because of perceived lower sustained virological response (SVR) rates and serious adverse events. We aimed to evaluate safety and efficacy of pegylated interferon alfa-2a and ribavirin therapy in elderly patients. Patients were stratified according to age (< 60 years *vs* ≥ 60 years). SVR rates showed an age related difference in patients with genotype 1 (23.7% *vs* 43.7%, *P* < 0.001) but not in genotype 2/3 infections (57.7% *vs* 64.6%, *P* = 0.341). Elderly hepatitis C virus patients differ in clinical characteristics and treatment outcome from younger patients and demand special attention from their practitioner.

INTRODUCTION

According to the estimation of the World Health Organization about 2% to 3% of the world's population is chronically infected with hepatitis C virus (HCV), which amounts to approximately 130-170 million people[1,2]. In Germany, community-based studies showed an overall prevalence of HCV-antibodies of about 0.5% in the adult population[3], representing 400000 to 500000 chronic infected people.

In western countries, contaminated blood products were the greatest risk factor for acquiring HCV infection before 1990, being replaced by intravenous drug abuse (IVDA) after 1990[4]. Nowadays IVDA remains a main risk factor together with sexual behavior (Men Who have Sex with Men) as well as tattooing. The number of iatrogenic infections is decreasing but infections still occur occasionally[5-9].

Since the rate of new infections has decreased over the last couple of years and the prevalence has peaked in western countries about a decade ago, the average age of the HCV patients has increased over the years[10].

The safety and efficacy of pegylated interferon (pegIFN) - based treatment regimens have been studied extensively[11,12] and have shown to reduce the risk of cirrhosis and hepatocellular carcinoma (HCC) and improve the survival of patients who achieve a sustained virological response (SVR)[13,14]. However, treatment is still relatively costly, burdensome for the patient and serious adverse events can occur[15]. The recent introduction of protease inhibitors for combination triple therapy has shortened average treatment duration and improved the

treatment outcome of patients with HCV genotype 1 infection considerably[16-18].

An increasing risk of liver cirrhosis and HCC development with advanced age has been repeatedly shown[10,19-22]. Hence, elderly patients are in special need of an effective antiviral treatment. However, co-morbidities like diabetes mellitus, co-medication and the risk of advanced liver fibrosis commonly found in older patients are known unfavourable factors for treatment outcome[23], others like coronary heart disease are important relative contra-indications for treatment[15]. In addition, adverse events and poor tolerability increase with age according to some studies[24]. As most clinical trials exclude patients above 65 years, safety and efficacy data for the treatment of older patients is limited. Thus, older patients as well as their attending physicians are often hesitant towards initiating a treatment course[25,26].

With the advent of novel treatment options providers have to weigh whether to initiate standard dual treatment in patients with genotype 1 or alternatively start with the novel triple therapy, which is associated with higher chance of attaining an SVR but also higher risk of serious adverse events. Alternatively, they might decide to defer treatment until after the introduction of interferon-free combination treatment options which are expected in the next couple of years[27,28]. However, due to the high cost of the novel direct antiviral agents pegIFN and ribavirin (RBV) will remain the standard of care in many countries.

German and European HCV guidelines give little or no guidance about until which age elderly patients should be treated nor even mention the issue of the ageing of HCV patients[29,30].

Only few studies with limited patient numbers and variable protocols studied the safety and efficacy of pegIFN and ribavirin in older patients with CHC[31-38]. Study results regarding SVR rates in elderly patients are inconsistent. Lower SVR rates and higher rates of adverse events and treatment discontinuation have been observed in most western studies[32,35-37,39]. These studies were mostly limited due to small patient numbers. In contrast, recently published Asian studies showed higher SVR rates in general[32,34,35] and a negligible influence of age on safety and efficacy[31,34]. The discrepancy of study results might be explained by distinct host genetic factors (such as a favourable IL28b polymorphism)[38].

The aim of the present study was to examine the safety and efficacy of pegIFN and ribavirin combination therapy in elderly patients in a large "real life" German cohort study.

MATERIALS AND METHODS

Patients

This analysis is part of an ongoing German multicenter non-interventional study (ML21645) of patients with CHC receiving pegylated interferon alfa-2a and RBV, involving 379 physicians/institutions throughout Germany

(328 in private practice and 51 in hospital settings). The study is ongoing since March 2003 and is approved by health authorities and ethical committees. Data of 4859 patients with completed documentation of treatment course at July 2011 were analysed. Recommended treatment duration was 24 wk for GT 2 and GT 3 infection and 48 wk for GT 1 and GT 4 infection. The treatment period was followed by an observational period of 24 wk. The recommended dosage of pegylated interferon alfa-2a was 180 μg once weekly in combination with RBV according to the SPCs of manufacturers. Inclusion criteria were age of at least 18 years, quantifiable HCV-RNA, compensated liver disease and written consent. SVR was defined as non-detectable HCV-RNA 24 wk after completion of the treatment period. Virological failure was defined as < 2 log decline of HCV RNA from baseline at week 12.

Data were obtained on structured questionnaires. Screening data included demographic data, history of HCV infection and concomitant diseases. During the treatment course information about virological response and drug safety was collected using online data entry. The study represents an unselected cohort in a real life setting including a significant fraction of all patients treated for hepatitis C mono-infection in Germany.

Because of limited numbers of liver biopsies for further assessment of liver fibrosis APRI (aspartate aminotransferase - platelet ratio index) was performed using pre-treatment laboratory data. APRI is a non-invasive indirect biochemical marker of hepatic fibrosis using routine laboratory parameters to distinguish fibrosis stages. APRI was calculated according to the formula proposed by Wai *et al*[40] in 2003: [(AST of the sample/reference AST) × 100]/platelets. Results were categorized as followed: ≤ 0.5 (no fibrosis); > 0.5-≤ 1.5 (mild fibrosis); > 1.5-≤ 2 (significant fibrosis); > 2 (cirrhosis).

Statistical analysis

The statistical analysis was descriptive to reflect the clinical routine as intended by the clinicians. Summary statistics (mean, median, standard deviation, 25th percentile, 75th percentile, minimum, maximum, number of values) or frequencies and proportions were assessed dependant on the scale level of the data.

Differences in baseline clinical characteristics, safety and efficacy data among the patient groups were compared statistically, using *t* test, χ^2 tests (Pearson and Fisher's exact test) and multivariate logistic regression analysis including OR and 95%CI.

All statistical analyses were based on 2-sided hypothesis tests. Analyses were calculated using SPSS for Windows Release 12.0.1, Testimate Version 6.4.27 and Matched Version 1.1.

RESULTS

Patient characteristics

Our analysis included 4859 patients infected with different HCV genotypes who were treated with a combination of pegylated interferon alfa-2a and a fix dosed or weight adjusted RBV. 4558 patients (93.8%) were < 60 years and only 301 patients (6.2%) were aged 60 years and older. Baseline characteristics, as shown in Table 1, differed in gender distribution, there were more women (55.8% *vs* 34.2%, *P* < 0.001) and predominantly GT 1 (81.4% *vs* 57.3%, *P* < 0.001) infected patients in the group of patients aged ≥ 60 years. There were no differences in racial distribution in both age groups with the vast majority of patients being caucasian. Providers assessed the fibrosis status more thoroughly in elderly patients and liver biopsies were performed more often in patients ≥ 60 years (29.9% *vs* 16.4%, *P* < 0.001). Liver biopsies displayed more advanced liver fibrosis in the older group (F3/4 31.1% *vs* 14.0%, *P* = 0.0003). For further assessment of fibrosis APRI was analysed for all patients. Approximately 30% of elderly patients showed significant fibrosis or cirrhosis (APRI ≥ 1.5) compared to 14.2% in the younger age group. Only 19.8% of elderly patients *vs* 43.3% of younger patients reached a score < 0.5 (Table 1).

As marker for liver synthesis values for International Normalized Ratio were categorized according to CHILD PUGH score. Only very few patients in both groups showed compromised coagulation. No significant difference was found in number of patients with pronounced coagulation disorder (Table 1).

As indirect markers of liver inflammation screening values of aspartate aminotransferase and alanine aminotransferase were analysed. AST was elevated in 83.7% of elderly patients and 63.5% of patients < 60 years with mean values of 92 U/L respectively 74 U/L. ALT was elevated in 90.5% of elderly patients and 80.0% of patients < 60 years, mean values 115 U/L respectively 107 U/L. Thrombocyte counts below normal range were seen in 25.4% of elderly patients compared to 11.5% in patients < 60 years (Table 1).

Comorbidities did not differ in number but in quality between both age groups. While metabolic (17.6%) and cardiovascular diseases (32.6%) dominated in patients ≥ 60 years, whereas drug and alcohol addiction (32.2%) as well as general psychological disorders (16.9%) were more frequent in patients < 60 years. The majority of older patients could not define the mode of HCV transmission. Main route of transmission in the group ≥ 60 years was blood transfusion (21.9%) followed by surgery (7.6%). In younger patients intravenous drug abuse was the most frequent reported transmission route (46.2%). Mean duration of infection was 19.9 years in older patients *vs* 11.6 years in younger patients (*P* < 0.001). The majority of patients in both age groups (older patients *vs* younger patients) were previously untreated. The percentage of untreated patients was slightly lower in the older age group (84.1% *vs* 88.6%, *P* = 0.02). HCV viral load prior to treatment stratified in low and high viral load (as defined by a cut of 400000 IE/mL) was not significantly different in either age groups (Table 1).

Safety

Generally HCV dual combination treatment with inter-

Table 1 Patient characteristics, transmission route and co-morbidities *n* (%)

Patients	< 60 yr	≥ 60 yr	*P* value
Mean age	4558 (94.0)	301 (6.0)	< 0.0001
Gender			< 0.0001
Male	3001 (65.8)	133 (44.2)	-
Female	1557 (34.2)	168 (55.8)	-
Race			
Caucasian	4373 (95.9)	289 (96.0)	
African	78 (1.7)	6 (2.0)	
Asian	88 (1.9)	5 (1.7)	
Hispanic	16 (0.4)	1 (0.5)	
Unknown/other	3 (0.1)	0	
Genotype			< 0.0001
1	2614 (57.3)	245 (81.4)	-
2	258 (5.7)	27 (9.0)	-
3	1480 (32.5)	21 (7.0)	-
Others (4, 5, 6)	206 (4.5)	8 (2.6)	
Initial viral load			
> 1 Mio copies/mL	2659 (58.9)	193 (64.5)	0.0830
Thrombocytes (/µL)	221.259 (635-595000; SD 71.884)	186.433 (21000-557500; SD 70546)	
Within normal range (140000-360000 c/µL)	3736 (85.1)	207 (72.9)	
Below normal range (< 140000 c/µL)	503 (11.5)	72 (25.4)	
Above normal range (> 360000 c/µL)	150 (3.4)	5 (1.8)	
GPT/ALT (U/L)	107 (4-1409; SD 103)	115 (20-591; SD 89)	
Within normal range (Male ≥ 50 U/L; Female < 35 U/L)	882 (20.0)	27 (9.5)	
Above normal range (Male ≥ 50 U/L; Female ≥ 35 U/L)	3521 (80.0)	258 (90.5)	
GOT/AST (U/L)	74 (10-2880; SD 87)	92 (18-526; SD 71)	
Within normal range (Male < 50 U/L; Female < 35 U/L)	1530 (36.5)	43 (16.3)	
Above normal range (Male ≥ 50 U/L; Female ≥ 35 U/L)	2658 (63.5)	221 (83.7)	
GGT (U/L)	96 (4-1871; SD 129)	111 (9-1240; SD 137)	
Within normal range (Male < 66 U/L; Female < 39 U/L)	2186 (50.2)	98 (35.4)	
Above normal range (Male ≥ 66 U/L; Female ≥ 39 U/L)	2165 (49.8)	179 (64.6)	
INR			
< 1.7	2758 (98.1)	182 (95.3)	
1.7-2.3	31 (1.1)	4 (2.1)	
> 2.3	22 (0.8)	5 (2.6)	
Body mass index (mean value)	25.2	26.5	0.000
Treatment naive	4038 (88.6)	253 (84.1)	0.0176
Estimated duration of infection (yr)	11.6	19.9	0.000
Histology available in Degree of Fibrosis	746 (16.4)	90 (29.9)	< 0.0001
P value adjusted (0-4 without unknown)			0.0003
F0/1/2	533 (71.4)	48 (53.3)	
F3/4	104 (14.0)	28 (31.1)	
Unknown	109 (14.6)	14 (15.6)	
APRI score			< 0.0001
< 1.5	3526 (85.7)	181 (70.2)	
≥ 1.5	587 (14.3)	77 (29.8)	
Duration of therapy (mean value) (wk)	32.5	33.0	0.6250
Co-morbidities (none/with)			0.6198
None	1792 (39.3)	114 (37.9)	-
With	2766 (60.7)	187 (62.1)	-
Cardiac	305 (6.7)	98 (32.6)	< 0.0001
Metabolic	204 (4.5)	53 (17.6)	< 0.0001
Drugs and alcohol	1469 (32.2)	10 (3.3)	< 0.0001
Psychogenic	768 (16.8)	26 (8.6)	< 0.0002
Skin	116 (2.5)	8 (2.7)	0.8500

Patient characteristics, transmission route and co-morbidities: multiple choice possible: comparison of patients < 60 years *vs* ≥ 60 years with chronic hepatitis. APRI score: [(aspartate aminotransferase (AST) of the sample/reference AST) × 100]/thrombocyte count. Cardiac: pAVK, coronary heart disease, stenocardia, cardiac infarction, arterial vascular disease, hypertension. Psychogenic: depression, psychosis, attempted suicide. Drugs and alcohol: Active, former and substituted consumption. Metabolic: Diabetes, disorder in lipid metabolism. ALT: Alanine aminotransferase; INR: International Normalized Ratio.

feron/ribavirin was relatively safe regardless of the age group. However, treatment had to be stopped more often in patients ≥ 60 years (47.8% *vs* 30.8%, *P* < 0.001). Main causes for treatment discontinuation in the older patients compared to younger patients were: virological failure [26.6% *vs* 13.6%, *P* < 0.001; OR = 2.291 (95%CI: 1.750-2.999)], adverse events [11.3% *vs* 3.1%, *P* < 0.001; OR = 3.960 (95%CI: 2.670-5.873)], patient request [7.3% *vs* 4.6%, *P* = 0.035; OR = 1.633 (95%CI: 1.035-2.575)], aggravating co-morbidities [2.3% *vs* 0.9%, *P* = 0.020; OR = 2.623 (95%CI: 1.167-5.898)], and death during therapy [1.0% *vs* 0.3%, *P* = 0.039; OR = 3.814 (95%CI: 1.070-13.588)], as depicted in Table 2.

Lack of compliance and lost-to-follow up were less common in older patients than younger patients (1.0% and 2.0% *vs* 3.5% and 7.1%).

The rates of drug modification are presented in Table 3. Patients ≥ 60 years had significant higher rates of dose modifications [30.9% *vs* 13.7%, *P* < 0.001; OR = 2.814 (95%CI: 2.172-3.644)] with reduction of both treatment components in 6.0% and reduction of just RBV and pegylated interferon alfa-2a, respectively, in 18.3% and 6.6% compared to 2.5% [*P* < 0.001; OR = 1.349 (95%CI: 1.138-1.600)], 6.3% [*P* < 0.001; OR = 1.827 (95%CI: 1.560-2.140)] and 4.9% (*P* = 0.183) in patients < 60 years.

Virologic responses

SVR was achieved in 94 of 301 patients ≥ 60 years and in 2230 of 4558 patients < 60 years (31.2% *vs* 48.9%, *P* < 0.001). Substratification for the different genotypes revealed that for GT-1 58 of 245 in elderly patients and 1142 of 2614 in younger patients achieved sustained virological response (23.7% *vs* 43.7%, *P* < 0.001). For GT 2 or GT 3 infections SVR rates were similar in both age groups (64.6% ≥ 60 years *vs* 57.7% < 60 years, *P* = 0.341) (Figure 1).

Table 2 Rates and reasons for treatment discontinuation *n* (%)

	All genotypes		*P* value	OR (95%CI)	Genotype 1		*P* value	OR (95%CI)
	< 60 yr	≥ 60 yr			< 60 yr	≥ 60 yr		
Treatment discontinuation	1402 (30.8)	144 (47.8)	0.000	2.065 (1.633-2.611)	985 (37.7)	134 (54.7)	0.000	1.996 (1.534-2.599)
Non-response	622 (13.6)	80 (26.6)	0.000	2.291 (1.750-2.999)	535 (20.5)	78 (31.8)	0.000	1.815 (1.365-2.414)
Lost to follow up	323 (7.1)	6 (2.0)	0.002	0.267 (0.118-0.603)	176 (6.7)	4 (1.6)	0.004	0.230 (0.085-0.625)
Adverse events	142 (3.1)	34 (11.3)	0.000	3.960 (2.670-5.873)	89 (3.4)	30 (12.2)	0.000	3.959 (2.558-6.126)
Of them due to intolerance RBV	49 (1.1)	19 (6.3)	0.000	6.200 (3.602-10.673)	35 (1.3)	16 (6.5)	0.000	5.148 (2.807-9.444)
Of them due to intolerance IFN	91 (2.0)	23 (7.6)	0.000	4.061 (2.530-6.519)	62 (2.4)	21 (8.6)	0.000	3.859 (2.309-6.448)
Patient request	210 (4.6)	22 (7.3)	0.035	1.633 (1.035-2.575)	127 (4.9)	21 (8.6)	0.013	1.836 (1.134-2.971)
Compliance	160 (3.5)	3 (1.0)	0.028	0.277 (0.088-0.872)	87 (3.3)	2 (0.8)	0.046	0.239 (0.058-0.977)
Co-morbidities	41 (0.9)	7 (2.3)	0.020	2.623 (1.167-5.898)	30 (1.1)	6 (2.4)	0.081	
Death	12 (0.3)	3 (1.0)	0.039	3.814 (1.070-13.588)	10 (0.4)	3 (1.0)	0.061	
Others	74 (1.6)	2 (0.7)	0.194		50 (1.9)	2 (0.8)	0.219	

Rates and reasons for treatment discontinuation: multiple choice possible: comparison of patients < 60 years *vs* ≥ 60 years with chronic hepatitis C. Others: Conspicuous blood results, hospitalisation, unexpected incident. RBV: Ribavirin; IFN: Interferon.

Table 3 Rates of dose modification *n* (%)

	All genotypes		*P* value	OR (95%CI)	Genotype 1		*P* value	OR (95%CI)
	< 60 yr (*n* = 4558)	≥ 60 yr (*n* = 301)			< 60 yr (*n* = 2614)	≥ 60 yr (*n* = 245)		
No dose reduction	3933 (86.3)	208 (69.1)	0.000	2.814 (2.172-3.644)	2204 (84.3)	166 (67.8)	0.000	2.558 (1.918-3.412)
Reduction of RBV	286 (6.3)	55 (18.3)	0.000	1.827 (1.560-2.140)	190 (7.3)	47 (19.2)	0.000	1.74 (1.460-2.074)
Reduction of pegINF α2a	224 (4.9)	20 (6.6)	0.183		129 (4.9)	17 (6.9)	0.173	
Reduction of RBV and pegINF α2a	115 (2.5)	18 (6.0)	0.001	1.349 (1.138-1.600)	91 (3.5)	15 (6.1)	0.039	1.218 (1.010-1.470)

Rates of dose modification: Comparison of patients < 60 years *vs* ≥ 60 years with chronic hepatitis C. RBV: Ribavirin; pegIFN: Pegylated interferon.
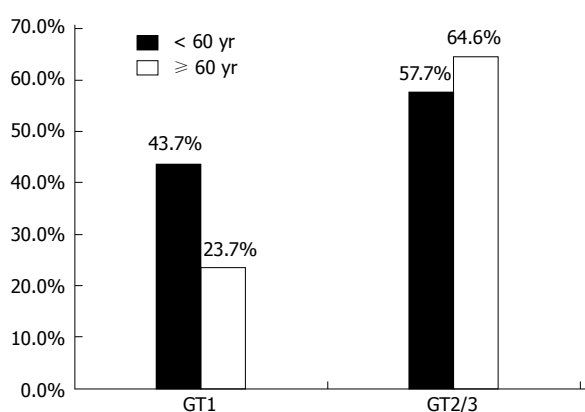
Figure 1 Sustained virological response rates in percent for different genotypes: comparison of patients < 60 years *vs* ≥ 60 years with chronic hepatitis C.

Table 4 Sustained virological response rates stratified for treatment history

		SVR				*P* value
		< 60 yr		≥ 60 yr		
All patients	GT 1	1142/2614	43.7%	58/245	23.7%	< 0.0001
	GT 2/3	1003/1738	57.7%	31/48	64.6%	0.3766
Treatment naïve	GT 1	1032/2250	45.9%	53/203	26.1%	< 0.0001
	GT 2/3	945/1608	58.8%	29/43	67.4%	0.2754
Treatment experienced	GT 1	110/364	30.2%	5/42	11.9%	0.0112
	GT 2/3	58/130	44.6%	2/5	40.0%	1

SVR: Sustained virological response.

Table 4.

In addition, SVR rates were stratified according to decades of age (Figure 2). For GT 1-infection a continuous decline in SVR rate with increasing decades of age was determined. In contrast, no significant difference in SVR rates for older patients with GT 2 and GT 3 was seen (Figure 2).

SVR rates were further stratified for APRI score < 1.5 *vs* ≥ 1.5 consistent with no or mild fibrosis *vs* severe fibrosis or cirrhosis. In elderly patients no significant dif-

Treatment naïve patients showed the same significant difference in treatment response in genotype 1 patients (26.1% ≥ 60 years *vs* 45.9% < 60 years, *P* < 0.001). SVR rates for GT2/3 patients were again similar in both age groups (67.4% ≥ 60 years *vs* 58.8% < 60 years, *P* = 0.341). Treatment experienced patients achieved significant lower SVR rates for all genotypes and age groups as shown in
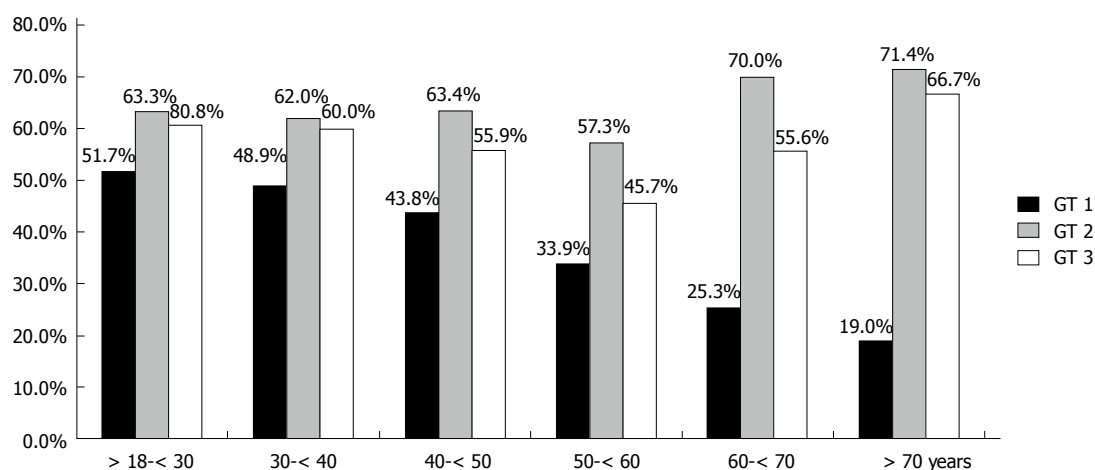
Figure 2 Sustained virological response rates in % for different age groups and different genotypes for patients with chronic hepatitis C.

ference in treatment response according to fibrosis stage was seen. In contrast, patients < 60 years showed significant lower SVR rates in patients with an APRI score ≥ 1.5. Data are consistent when stratified for GT 1 and GT 2/3 (Table 5).

Age and stage of liver disease in contrast to treatment discontinuation due to ribavirin and PEG-IFN adverse events were independent factors of SVR rates as shown in a multivariate logistic regression analysis (Table 6).

DISCUSSION

Despite the enormous data set and experience we have generated for standard combination interferon treatment for chronic HCV infection over the last decade, the growing population of elderly patients is a relatively understudied population. Many of the major registration trials excluded patients aged > 65 years. Also, clinical guidelines give no detailed advice for treatment of the elderly patient group[29,30], which is generally regarded as difficult to treat population due to higher rates of fibrosis and co-morbidities.

In this substudy of this ongoing German multicenter non-interventional study we evaluate the safety and efficacy of a combination therapy with pegylated interferon alfa-2a and RBV in HCV-positive patients ≥ 60 years in comparison to patients < 60 years.

Notably, only 6.2% (301/4859) of all treated patients were ≥ 60 years suggestive of a relative under-treatment of elderly HCV-infected patients. As our study only included patients in whom treatment was initiated no data of treatment uptake rates for the different age groups are available. For previous analyses of the ongoing study data of all patients screened for possible HCV therapy was obtained. A first epidemiological study showed a high percentage of elderly patients in the group of all HCV patients with 26.3% (2716/10326) of the patients being ≥ 60 years old[41]. A second study showed a significant lower rate of treatment uptake in patients > 56 years compared to patients ≤ 56 years (28.2% *vs* 49%) -

in patients aged between 65 and 70 years treatment rate was 26.3%[42]. An Italian cross-sectional study and the Veterans Affairs Medical Centres Study both showed that advanced age is often the main reason to exclude elderly patients from treatment[25,26]. Additional studies are needed to evaluate the general treatment assessment of elderly patients and further characterize the group of patients who are a priori excluded from treatment.

Consistent with data from other Western countries[36,37,43] patient's characteristics differed significantly in gender, genotype, co-morbidities and transmission risk. Patients ≥ 60 years were rather female, more likely infected with GT 1, suffering from metabolic or cardiovascular diseases and being infected iatrogenic. Not surprisingly, older patients showed more advanced liver disease. As histology was assessed only in about one third of the patients APRI score was performed for all patients. Data were consistent with the results of histology with about 30% of patients in the elderly group reaching a score of > 1.5 consistent with significant fibrosis or cirrhosis. On the other hand, approximately 20% of the older patient cohort did show little or no signs of fibrosis. The benefit of treating these patients is not so clear, especially in the absence of symptoms in some of these patients. In a Japanese cohort study only patients with a reduced platelet count as marker of advanced fibrosis showed significantly differences in hepatocarcinogenesis and survival compared to an untreated reference group[44].

Elderly patients had a significantly higher rate of treatment discontinuation (47.8% *vs* 30.8%, *P* < 0.001). The main reason was non-response (26.6%).

In 11.3% treatment was interrupted due to adverse events, another 7.3% of the patients requested premature discontinuation. Surprisingly, despite a high rate of metabolic and cardiovascular comorbidities in the elderly, treatment was stopped due to worsening of these underlying diseases only in 7/301 (2.3%) patients. 3 patients (1%) in the elderly patients group died during therapy - 2 of them due to complications of liver cirrhosis, the other patient due to deterioration of general condition.

Table 5 Sustained virological response rates stratified for genotype, APRI score and age

	APRI score	SVR *n* (%)	*P*
GT 1			χ^2 test
< 60 yr	< 1.5	936 (45.7)	0.000
	≥ 1.5	99 (31.2)	
≥ 60 yr	< 1.5	40 (26.5)	0.476
	≥1 .5	12 (20.7)	
GT 2/3			
< 60 yr	< 1.5	799 (60.8)	0.000
	≥ 1.5	106 (43.6)	
≥ 60 yr	< 1.5	18 (66.7)	0.526
	≥ 1.5	9 (52.9)	

SVR: Sustained virological response.

Table 6 Multivariate logistic regression for fibrosis, age and ribavirin/peg-interferon adverse events as factors for sustained virological response

	Sig.	Exp (B)	95%CI for EXP(B)	
			Lower	Upper
Fibrosis stage F4 *vs* Fibrosis stage F0, F1, F2, F3	0.004	0.717	0.573	0.897
Age 60 yr *vs* < 60 yr	0.003	0.427	0.243	0.750
Treatment discontinuation due to ribavirin adverse events	0.142			
Treatment discontinuation due to Peg-IFN adverse events	0.998			

IFN: Interferon.

Mortality rate in the younger patients was slightly lower with 12/4558 (0.3%). Causes of death in the younger age group were infectious complications, drug overdose and suicide, cardiovascular disease, liver failure and pulmonary embolism. The slightly higher mortality rate in elderly patients might be probably due to the low number of patients as well as due to the general higher mortality in advanced age. Treatment adherence was slightly higher in older patients, compliance problems occurred in only about 1% *vs* 3.5% in younger patients and only 2% *vs* 7.1% were lost to follow-up.

As reported before[31-33], we also noted more dose modifications during HCV therapy in elderly compared to younger patients. It is not entirely clear whether these modifications were always justified or whether the providers acted with extra care for fear of adverse events, *e.g.* cardiac ischemia due to anaemia. In nearly one third of the patients the initial dose of one or both drugs had to be reduced during the treatment course. In 24.3% of older patients RBV dose was reduced.

Treatment response in patients with GT 1 was substantially lower in patients ≥ 60 years (23.7% *vs* 43.7%, *P* < 0.001). Furthermore, we found a continuous decline in sustained virological response rates over age for GT 1 infections when classifying them into groups by decades (Figure 2). Consistent with recently published data[31,34,36], no significant difference in the SVR rates was found for GT 2 and GT 3 infections. As expected, treatment response was substantially lower for retreated patients. 5 out 42 (11.9%) elderly patients with GT 1 infection achieved SVR. In GT 2/3 patients only 5 patients were pre-treated, two of them were retreated successfully. Stratified for stage of fibrosis advanced fibrosis (APRI score > 1.5) was clearly associated with a lower treatment response in patients < 60 years both for GT 1 and GT2/3 infections. No such correlation was seen in the group of elderly patients. Still lower SVR rates were found for APRI score 1.5 but the results were not statistically significant which might mainly explained due to the small patient numbers. Age and stage of liver disease (fibrosis stage 4 *vs* other fibrosis stage) could be shown as independent factors of treatment response in multivariate regression analysis.

Recently published data of treatment outcome in elderly patients with pegylated IFN based regimens showed consistently higher SVR rates ranging from 40.7%[34] up to 67.1%[31] for GT 1 infections and 76.7%[31] and 86.4%[34] for GT 2 or GT 3. Both studies were conducted in Asian patients who generally show higher response rates compared to Caucasians and Afro-Americans mainly due to host genetic variations *e.g.*, the recently described IL28B polymorphisms[38].

The relatively low SVR rates in older patients are mainly caused by higher rates of virological non-response to dual therapy, which might be due to the difference in quality not in quantity of comorbidities as well as advanced liver fibrosis. But still, it could be shown that age is an independent factor for SVR. The reason remains unknown. Altered IFN-immunomodulation and pharmacokinetics in elderly patients might influence the response to therapy. Their affect has to be evaluated further. These factors might even become more apparent with longer duration of therapy and may explain that age-dependent differences in SVR rates seen in patients with genotype 1 infections in contrast to similar SVR rates in all age groups for genotype 2 and 3 infections in whom duration of therapy is markedly lower.

The recently approved new direct antiviral agents such as protease inhibitors and polymerase inhibitors might provide more effective treatment options. Triple therapy regimens with the protease inhibitors telaprevir or boceprevir have to be considered for many elderly GT 1 patients despite the possibility of further side-effects. Both drugs showed only slightly lower SVR rates in patients > 40 years compared to patients < 40 years in phase III-trials[16,17]. But the clinical studies did not include sufficient numbers of patients ≥ 60 years to prove the superior efficacy for this age group.

Furthermore, protease inhibitors may hold new obstacles such as drug drug interactions with concomitant medication. Adverse events like anaemia and rash will require an even more intense monitoring of the patient during treatment course[45,46]. Studies to assess the safety and efficacy of triple therapy in older patients are ur-

gently needed. The low SVR rates of dual therapy and possible complications of protease inhibitor based triple therapy might indeed be an argument for postponement of treatment in some patients until the interferon free regimens will become widely available[27,28]. The nucleotide NS5B polymerase inhibitor Sofosbuvir has been approved for HCV therapy by FDA in the end of 2013 offering the first IFN-free treatment alternative for patients with genotype 2 or 3 infections and those with contraindications against interferon[47-49].

In contrast, the SVR rates remain high for GT 2 and GT 3 patients even with increasing age but small patient numbers have to be taken into account. Still, our findings are consistent with previously published data[31,34,36]. In respect of SVR rates of up to 65% regardless of age, short treatment duration and relative low cost of dual treatment, there is less of a rationale to postpone treatment until the introduction of intensified treatment regimens or interferon-free combination treatment.

We conclude that the elderly HCV patient is still understudied and not well understood. National and European guidelines should take into account the general ageing of HCV patients in Europe. Despite higher rates of treatment discontinuation and lower SVR rates in GT 1 infection, HCV-therapy in elderly patients is well feasible in "real life" experience. Therefore elderly patients should not be excluded from assessment for treatment a priory. We suggest making informed decisions on individual basis and taking all of the patient's circumstances into account, such as stage of liver disease, clinical symptoms and comorbidities as well as virological parameters, treatment history and further predictive parameters. New therapy regimens containing more potent direct antiviral agents may enhance treatment outcome in elderly patients but further studies to examine the effect of age on safety and efficacy of these agents are urgently needed. Still, pegylated interferon based dual therapy will remain the standard of care for treatment of hepatitis C in many countries to the immense cost of the novel direct antiviral agents.

COMMENTS

Background
The average age of hepatitis C virus (HCV) patients is increasing over time. There are concerns to initiate treatment in elderly patients because of perceived lower sustained virological response (SVR) rates and serious adverse events. As elderly patients were excluded from most clinical trials in the past, safety and efficacy data for the treatment of elderly patients is limited. Therefore, the authors aimed to evaluate safety and efficacy of pegylated interferon alfa-2a and ribavirin therapy in patients > 60 years.

Research frontiers
The safety and efficacy of pegylated interferon - based treatment regimens in patients with hepatitis C infection have been studied extensively and have shown to reduce the risk of cirrhosis and hepatocellular carcinoma and improve the survival of patients who achieve a sustained virological response. However, only few studies with limited patient numbers and variable protocols studied the safety and efficacy of pegylated interferon and ribavirin therapy in elderly patients. Furthermore, study results regarding SVR rates in elderly patients are inconsistent.

Innovations and breakthroughs
The study represents an unselected cohort in a real life setting including a significant fraction of all patients treated for hepatitis C mono-infection in Germany providing safety and efficacy data on pegylated interferon and ribavirin therapy in 301 patients > 60 years.

Applications
The study highlights that elderly HCV patients differ in clinical characteristics and treatment outcome from younger patients and that they demand special attention from their practitioner. Still, despite higher rates of treatment discontinuation and lower SVR rates in GT 1 infection, HCV-therapy in elderly patients is well feasible in "real life" experience. Therefore elderly patients should not be excluded from assessment for treatment a priory. Informed decisions should be made on individual basis and taking all of the patient´s circumstances into account, such as stage of liver disease, clinical symptoms and comorbidities as well as virological parameters, treatment history and further predictive parameters.

Peer review
The authors of this study present a report on HCV treatment efficacy and safety/tolerability in elderly patients. The subject is important and the authors performed a good study on this subject. The paper is original, very interesting and very well-written.

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OBSERVATIONAL STUDY

Clinical and endoscopic characteristics of drug-induced esophagitis

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Abstract

AIM: To investigate clinical, endoscopic and pathological characteristics of drug-induced esophagitis.

METHODS: Data for patients diagnosed with drug-induced esophagitis from April 2002 to May 2013 was reviewed. Patients diagnosed with malignancy, viral or fungal esophagitis were excluded. Clinical, endoscopic and pathological characteristics of patients diagnosed with drug-induced esophagitis were analyzed.

RESULTS: Seventy-eight patients were diagnosed with drug-induced esophagitis. Their mean age was 43.9 ± 18.9 years and 35.9% were male. Common symptoms were chest pain (71.8%), odynophagia (38.5%) and dysphagia (29.5%). The endoscopic location was in the middle third of esophagus in 78.2%. Endoscopic findings were ulcer (82.1%), erosion (17.9%), ulcer with bleeding (24.4%), coating with drug material (5.1%), impacted pill fragments (3.8%) and stricture (2.6%). Kissing ulcers were observed in 43.6%. The main causative agents were antibiotics and non-steroidal anti-inflammatory drugs. All the patients were treated with proton pump inhibitors (PPIs) or sucralfate, and the causative drugs were discontinued. Nineteen patients with drug-induced esophagitis were followed up with endoscopy and revealed normal findings, scars or healing ulcers.

CONCLUSION: Drug-induced esophagitis mainly presents as chest pain, odynophagia and dysphagia, and may be successfully treated with PPIs and discontinuation of the causative drug. Kissing ulcers were observed in 43.6%.

Core tip: This study investigated the clinical characteristics of drug-induced esophagitis, such as the main symptoms, common endoscopic findings and main causative agents. Uniquely, kissing ulcers were observed in 43.6% of drug-induced esophagitis, which is

a higher rate than in the previous reports. This might be helpful in diagnosing this rare disease. To the best of our knowledge, the present study is the first to compare the histopathological features between drug-induced esophagitis group and reflux esophagitis group.

INTRODUCTION

To date, hundreds of drugs have been reported to cause drug-induced esophagitis. However, many clinicians do not recognize this as a cause of chest pain or odynophagia. The majority of the patients usually report self-limited symptoms, so this diagnosis is often underestimated[1]. However, lack of awareness of drug-induced esophagitis can lead to persistent exposure to causative drugs, resulting in severe complications[2-4]. Patients who are not initially and accurately diagnosed with drug-induced esophagitis may suffer from unnecessary work-up or extensive diagnostic evaluation for chest symptoms. To avoid these undesirable situations, awareness of this disease must be improved. Nonetheless, most of the studies on drug-induced esophagitis are case reports or reviews of case reports, which provide limited understanding of this disease. The purpose of this study was to investigate the clinical and endoscopic characteristics of drug-induced esophagitis.

MATERIALS AND METHODS

Study population

The data for 78 patients diagnosed with drug-induced esophagitis between April 2002 and May 2013 was reviewed and analyzed from four university hospitals. Patients with a definite history of taking medicines and with acute esophageal symptoms (odynophagia, dysphagia and chest pain) of less than two weeks were included in the drug-induced esophagitis group. Demographic features, clinical history, endoscopic findings and histopathological features were obtained by reviewing electronic medical records at each hospital. Patients with malignancy, viral or fungal esophagitis, esophageal varix, and corrosive esophageal injury were excluded. Patients with esophageal reflux symptoms that were persistent for greater than two weeks were also excluded. To compare their histopathology with the drug-induced esophagitis group, 19 patients with endoscopic evidence of reflux esophagitis (grade A to D according to the Los Angeles classification) and gastrointestinal symptoms were selected and included in the reflux esophagitis group[5]. The Institutional Review Board of Seoul National University Boramae Hospital approved the study, which was performed in accordance with the ethical guidelines of the Declaration of Helsinki.

Statistical analysis

SPSS version 18.0 software (IBM, Chicago, IL, United States) was used for statistical analysis. Continuous data were tested for the normality assumption using the Kolmogorov-Smirnov test. Normally distributed variables were described using the mean and SD. Descriptive data were shown as mean ± SD, number of patients and percentage. Categorical variables were analyzed between groups using the χ^2 test. All results were considered statistically significant when P values were less than 0.05 (two-tailed).

RESULTS

Demographic findings and clinical symptoms

Among 78 patients with drug-induced esophagitis, 35.9% ($n = 28$) were males and 64.1% ($n = 50$) were females. Their mean age was 43.9 ± 18.9 years (mean ± SD, range 16-84).

Common symptoms were chest pain ($n = 56$, 71.8%), odynophagia ($n = 30$, 38.5%), dysphagia ($n = 23$, 29.5%) and vomiting ($n = 6$, 7.7%). Two patients had melena ($n = 2$, 2.6%) caused by esophageal bleeding (Table 1).

Endoscopic findings

78.2% (61/78) of the endoscopic location of drug-induced esophagitis was in the middle third of the esophagus. Endoscopic findings in the esophagus were ulcers ($n = 64$, 82.1%), erosions ($n = 14$, 17.9%), ulcer with bleeding ($n = 19$, 24.4%), coating with drug material ($n = 4$, 5.1%), impacted pill fragments ($n = 3$, 3.8%) and stricture ($n = 2$, 2.6%). Thirty-four cases (43.6%) showed kissing ulcers (ulcers facing each other) (Figure 1, Table 2).

Causative agents

Causative agents were antibiotics (doxycycline, amoxicillin, ciprofloxacin, metronidazole, sultamicillin tosylate and rifaximin) in 28 patients (35.9%), non-steroidal anti-inflammatory drug (as) (aspirin, aceclofenac) in 27 patients (34.6%), anti-hypertensive drugs (amlodipine, ramipril) in nine patients (11.5%), acetaminophen in seven patients (9.0%), oral hypoglycemic agents (glimepiride) in four patients (5.1%), bisphosphonates (alendronate, ibandronate) in four patients (5.1%), ascorbic acid in 2 patients (2.6%), warfarin in 2 patients (2.6%) and other drugs (tiropramide, pinaverium bromide, mosapride, esomeprazole) in 4 patients (Table 3). The proportion of antibiotics as a cause of drug-induced esophagitis was higher among the younger group (< 45 years) than in the elderly group ($\geqslant$ 45 years, 47.6% *vs* 22.2%, $P = 0.02$, χ^2 test). The proportion of NSAID as a cause of drug-induced esophagitis showed no significant differences between the two age groups (28.6% *vs* 41.7%, $P = 0.226$, χ^2 test) (Table 4).

Table 1 Demographic features and clinical symptoms of patients diagnosed with drug-induced esophagitis *n* (%)

Characteristics		
Age(yr)	mean ± SD	43.9 ± 18.9
Sex	Male/female	28/50
Symptom	Chest pain	56 (71.8)
	Odynophagia	30 (38.5)
	Dysphagia	23 (29.5)
	Vomiting	6 (7.7)
	Melena	2 (2.6)

Table 2 Endoscopic features of patients diagnosed with drug-induced esophagitis

Feature		*n* (%)
Location	Proximal	3 (3.8)
	Middle	61 (78.2)
	Distal	14 (17.9)
Endoscopic findings	Ulcers	64 (82.1)
	Bleeding	19 (24.4)
	Erosions	14 (17.9)
	Coating	4 (5.1)
	Pill	3 (3.8)
	Stricture	2 (2.6)
	Kissing ulcers	34 (43.6)

Pathological findings

In 17 cases (21.8%), endoscopic biopsy was performed to evaluate the pathological finding of the esophageal lesion. Pathological findings were evaluated between the drug-induced esophagitis group and the reflux esophagitis (RE) group. There were no significant differences in basal cell hyperplasia ($P = 0.559$), papillary elongation ($P = 0.086$), dilated intercellular spaces ($P = 0.175$), and cell vacuolization ($P = 0.074$) between the two groups (Table 5).

Treatment and follow up

All of the patients were treated with proton pump inhibitors (PPIs) or sucralfate and the causative drugs were discontinued. Nineteen patients (24.4%) with drug-induced esophagitis were followed up with endoscopy after 2 d-2 mo, where they revealed normal findings or well-healed scars in the esophagus in all but two patients who still had healing ulcers. The remaining 59 patients (75.6%) had no symptoms during follow up and did not undergo follow up endoscopy or were lost during follow up.

DISCUSSION

If impacted pill fragments are present in the esophagus during the endoscopic examination of a symptomatic patient, a clear diagnosis can be made. However, impacted pill fragments are rarely found. Pathological findings, such as brown-black crystals for iron, and basophilic crystals for Kayexalate, are known to aid in diagnosing drug-induced esophagitis. Mitotic arrest is also a pathological finding helpful in diagnosing drug-induced esophagitis caused by taxol or colchicines. Other than these reported rare cases, diagnosing drug-induced esophagitis is based on the clinical history and endoscopic findings. Many cases reporting drug-induced esophagitis were identified. However, other than case reports, there were very few studies addressing the characteristics of drug-induced esophagitis[6,7]. Higuchi *et al*[8] reported that the etiologies of esophageal ulcers included RE in 65.9%, drug-induced esophagitis in 22.7% and the others (viral, fungal *etc*.) in 11.4%. When esophageal ulcers are encountered during endoscopy, reflux esophagitis or drug-induced esophagitis should first be considered, given that there is no clinical suspicion of other diseases (*i.e.*, viral/fungal esophagitis, Levin tube injury, Crohn's disease, or radiation injury). Higuchi *et al*[8] also reported that 91.4% of

RE-induced esophageal ulcers were located in the lower esophagus and 80% of drug-induced esophageal ulcers were located in the middle portion of the esophagus. Other studies also found that lesions of drug-induced esophagitis were frequently located in the middle third of esophagus[6,7]. The middle third of the esophagus is subject to compression by the aortic arch or enlarged left atrium; therefore, drug-induced esophagitis is commonly located in the mid-esophagus[9]. Therefore, with the location of esophageal ulcers, RE can be differentiated from drug-induced ulcers in many cases. Typical reflux esophagitis patients often have persistent reflux symptoms and patients with drug-induced esophagitis, in general, have abrupt-onset chest symptoms. According to Kirkendall, the typical drug-induced esophagitis patient presents with the sudden onset of odynophagia, dysphagia or retrosternal pain[10]. Based on this report, the study of Abid *et al*[6] was performed with patients who experienced acute onset of esophageal symptoms of less than 3 d duration. According to Boyce, symptoms of drug-induced esophagitis can develop within hours to 10 d after medication[11]. After being lodged in the esophagus, injurious pills release noxious contents damaging the esophageal wall[10]. Thus, it is postulated that this damage of esophageal wall gives rise to the abrupt-onset symptoms of drug-induced esophagitis. Patients with drug-induced esophagitis often have a history of medication in the recumbent position or before going to sleep, with no or little water[10,12]. In our study, patients with a definite history of taking medicines and with acute esophageal symptoms of less than two weeks were included.

As eosinophilic infiltration is frequently found in the distal esophagus of reflux esophagitis; mid-to-proximal esophagus is recommended for tissue biopsy of eosinophilic esophagitis[13]. The location of the lesions in eosinophilic esophagitis is similar to drug-induced esophagitis and eosinophilic infiltration is also commonly found in drug-induced esophageal lesions[14]. Therefore, a differential diagnosis between eosinophilic esophagitis and drug-induced esophagitis can be unclear. Though most patients with eosinophilic esophagitis have abnormal endoscopic findings, endoscopic changes alone are inadequate for the diagnosis of eosinophilic esophagitis[15]. The differentiation between eosinophilic esophagitis and drug-induced
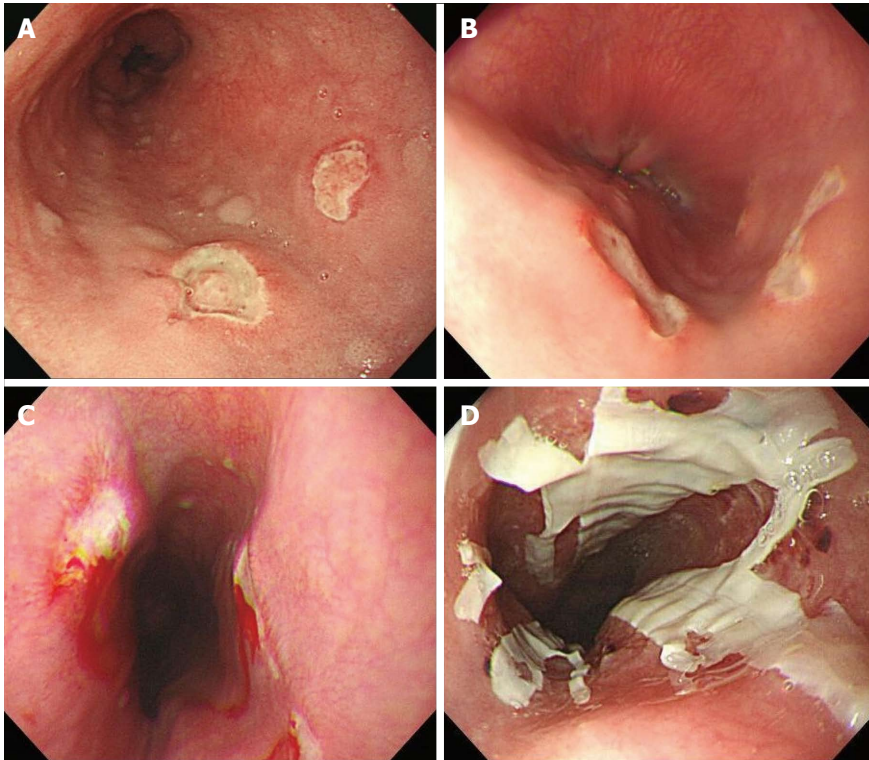
Figure 1 Endoscopic findings of drug-induced esophagitis. A: Typical kissing ulcers in the middle third of esophagus; B: Another typical kissing ulcer; C: Kissing ulcers with spontaneous bleeding; D: Coating with drug material.

Table 3 Causative drugs of patients diagnosed with drug-induced esophagitis

Drug	*n* (%)
Antibiotics	28 (35.9)
NSAID	27 (34.6)
Anti-hypertensive	9 (11.5)
Acetaminophen	7 (9.0)
Oral hypoglycemic	4 (5.1)
Bisphosphonate	4 (5.1)
Ascorbic acid	2 (2.6)
Warfarin	2 (2.6)
Other drugs	4 (5.1)

NSAID: Non-steroidal anti-inflammatory drug.

Table 4 Proportion of antibiotics and non-steroidal anti-inflammatory drugs between both age groups

	Age		Total	*P* value
	< 45 yr	≥ 45 yr		
Antibiotics				0.020
(+)	20	8	28	
(-)	22	28	50	
Total	42	36	78	
NSAID				
(+)	12	15	27	0.226
(-)	30	21	51	
Total	42	36	78	

NSAID: Non-steroidal anti-inflammatory drug.

esophagitis needs to be a clinicopathological diagnosis, which requires clinical findings and pathological criteria for a diagnosis[16]. In differentiating the diagnosis of eosinophilic esophagitis and reflux esophagitis, endoscopic findings and clinical response to medication of reflux esophagitis can be useful[14]. There are some studies on histological parameters for the differential diagnosis of eosinophilic esophagitis and reflux esophagitis[14,17]. Our study attempted to find pathological clues that can differentiate drug-induced esophagitis from reflux esophagitis; however, there were no significant differences of basal cell hyperplasia ($P = 0.559$), papillary elongation ($P = 0.086$), dilated intercellular spaces ($P = 0.175$) and cell vacuolization ($P = 0.074$) between the two groups. To the best of our knowledge, the present study is the first study

to compare the histopathological features between a drug-induced esophagitis group and a reflux esophagitis group.

There are reports that drug-induced esophagitis is predominantly found among elderly patients, as they are more likely to spend time in the recumbent position, consume more medications, including alendronate or non-steroidal anti-inflammatory drugs (NSAIDs), have more esophageal motility problems or cardiac enlargement with mid-esophagus compression, and are less aware of the drug instructions[11]. A study showed that the esophageal transit time was significantly longer in elderly subjects than in younger subjects[18]. However in our study, the proportion of antibiotics use was higher in younger group than in elderly group. According to the literature, antibiotics were the commonest or second commonest cause of

Table 5 Pathological findings of drug-induced esophagitis group and reflux esophagitis group *n* (%)

	Drug-induced esophagitis (*n* = 17)	Reflux esophagitis (*n* = 19)	*P* value
Basal cell hyperplasia	6 (35.3)	5 (26.3)	0.559
Papillary elongation	5 (29.4)	11 (57.9)	0.086
Dilated intercellular spaces	11 (64.7)	8 (42.1)	0.175
Cell vacuolization	13 (76.5)	9 (47.4)	0.074

drug-induced esophagitis[6,9]. In our study, antibiotics were the commonest causative drugs. In contrast to NSAIDs, anti-hypertensive drugs and bisphosphonates, which are frequently prescribed for elderly patients, antibiotics are commonly prescribed in young patients to treat acne, urinary tract infections or pelvic inflammatory disease[11]. Our study showed that the predominant causative drugs were different between age groups. Previous reports showed that drug-induced esophagitis was more prevalent among women than among men[1,6]. In this study, 64.1% were females, which was consistent with previous reports.

Our study showed that the common symptoms were chest pain, odynophagia and dysphagia. Many of these patients reported multiple symptoms, such as odynophagia with concurrent chest pain. Zografos *et al*[1] showed that the main symptoms caused by drug-induced esophagitis were chest pain (60%), odynophagia (50%), and dysphagia (40%). 78.2% of endoscopic locations of drug-induced esophagitis were found in the middle third of esophagus, which was consistent with previous studies[6,8]. In thirty-four cases (43.6%), there were kissing ulcers (ulcers facing each other). Kissing ulcers were also reported in esophageal injury other than drug-induced esophagitis[19]. Therefore, kissing ulcers alone cannot confirm drug-induced esophagitis. However, we showed that kissing ulcers were observed in drug-induced esophagitis more frequently than the previously reported studies[6]. Patients with longer esophageal symptoms were included in our study; therefore, the duration of esophageal exposure to causative agents may be longer. This may have contributed to the formation of kissing ulcers. A clinical study on drug-induced esophagitis showed that kissing ulcers occupied 7.6%, which is lower than in our study[6]. In Higuchi's study, active bleeding was noted in 45% of drug-induced esophageal ulcers, which is higher than the 24.4% in our study[8]. This difference can be explained by the difference in the proportion of patients taking NSAIDs (65% *vs* 34.6%). Notably, the study of Higuchi *et al*[8] included only esophageal ulcers, whereas our study included shallow esophageal erosions, as well as esophageal ulcers. From these results, drug-induced esophagitis should also be considered as a cause of upper gastrointestinal bleeding. Two cases with esophageal stricture were also identified, both of which had dysphagia symptoms and were associated with NSAID use. It has been reported that NSAIDs were associated with an increased risk of reflux esophagitis and esophageal strictures[20]. In

patients with reflux esophagitis, one should be careful in prescribing NSAIDs. It was reported that pill fragment impaction was associated with esophageal stricture[21]. Here, we observed three cases of impacted pill fragments with no definite esophageal stricture.

For patients with drug-induced esophagitis, oral sucralfate and PPIs are frequently administered, and the offending drugs are discontinued[6]. In our study, 19 patients (24.4%) with drug-induced esophagitis were treated with oral sucralfate, PPI and quitting drugs. These patients were then, followed up with endoscopy after 2 d-2 mo; where most of them revealed normal findings or well-healed scars in the esophagus, and only two patients still had healing ulcers. Once the offending drug is discontinued, oral sucralfate and PPI are thought to be sufficient for the treatment of drug-induced esophagitis. Intramural esophageal hematoma with drug-induced esophagitis was also reported to have a favorable outcome after a conservative treatment[22]. In contrast, it has been reported that endoscopic intervention was necessary to treat complications of drug-induced esophagitis[23].

If a medication history and chronology of acute esophageal symptoms strongly suggest it, diagnosing drug-induced esophagitis is not so difficult, even without endoscopic examination[11]. However, the diagnosis of drug-induced esophagitis can be more easily confirmed with the appropriate endoscopic findings. Additionally, helpful findings, such as pill fragments or residues can be observed at the sites of injury, making the diagnosis clear[24]. Malignancy and viral or fungal esophagitis can also be ruled out using endoscopy.

This study is a retrospective observational study, and lacks a control group. Therefore, it is difficult to measure the significance of the descriptive results. However, from the results of our study with 78 subjects, the clinical characteristics such as main symptoms, common endoscopic findings (ulcers in the middle third of esophagus) and main causative agents could be identified. A unique finding in this study was that kissing ulcers were observed in 43.6% of the patients diagnosed with drug-induced esophagitis, which might be helpful in diagnosing this rare disease.

In conclusion, drug-induced esophagitis mainly presented as chest pain, odynophagia and dysphagia, and was successfully treated with PPIs and the discontinuation of the causative drug. Kissing ulcers were observed in 43.6% of the patients diagnosed with drug-induced esophagitis. It is important to be mindful of the possibility of drug-induced esophagitis in patients with acute esophageal symptoms. With an accurate diagnosis, patients will be able to avoid unnecessary work-up or fatal complications.

COMMENTS

Background
Drug-induced esophagitis is a rare disease, and the likelihood of this diagnosis is often underestimated. Lack of awareness of drug-induced esophagitis can lead to severe complications or unnecessary work-up.

Research frontiers
Most studies on drug-induced esophagitis are case reports or reviews of case reports, and large-scale studies are rare. In this study, the authors investigated

the clinical and endoscopic characteristics of drug-induced esophagitis in a multi-center setting.

Innovations and breakthroughs

A unique finding was that kissing ulcers were observed in 43.6% of the patients diagnosed with drug-induced esophagitis, which might aid in diagnosing this rare disease. This study is also the first study to compare the histopathological features between a drug-induced esophagitis group and a reflux esophagitis group.

Applications

Clinical characteristics such as symptoms, common endoscopic findings and main causative agents were identified. The main symptoms were chest pain, odynophagia, and dysphagia. Common endoscopic findings were ulcers in the middle third of esophagus; kissing ulcers were frequently observed. These findings could be helpful in the diagnosis of drug-induced esophagitis.

Terminology

Drug-induced esophagitis is a clinical problem caused by esophageal damage associated with the ingestion of certain drugs. Kissing ulcers are ulcers facing each other, which is a common finding in drug-induced esophagitis, though it is not pathognomonic. Non-steroidal anti-inflammatory drugs are drugs, including aspirin and ibuprofen, which are used for reducing inflammation and pain in various diseases. Proton pump inhibitors are drugs that irreversibly inhibit proton pump function and are the most potent gastric acid-suppressing agents in clinical use.

Peer review

This is a very interesting observational study on the clinical, endoscopic and pathological characteristics of drug-induced esophagitis. From the results of this study, practitioners can identify the features of drug-induced esophagitis and also get help in diagnosing patients with drug-induced esophagitis.

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RANDOMIZED CONTROLLED TRIAL

Randomized controlled trial: Moxibustion and acupuncture for the treatment of Crohn's disease

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Abstract

AIM: To evaluate the clinical efficacy and safety of acupuncture and moxibustion for the treatment of active Crohn's disease (CD).

METHODS: Ninety-two patients were equally and randomly divided into the treatment group and received herb-partitioned moxibustion combined with acupuncture, and the control group received wheat bran-partitioned moxibustion combined with superficial acupuncture. The patients received three treatment sessions per week for 12 wk and were followed up for 24 wk. The main outcome was evaluated using the CD Activity Index (CDAI) score, and the secondary outcomes were evaluated using laboratory indicators such as hemoglobin (HGB), C-reactive protein (CRP), erythrocyte sedimentation rate, quality-of-life, endoscopic ratings, and intestinal histology scores.

RESULTS: The CDAI scores of both the treatment and control groups were significantly reduced after treatment compared with those measured before treatment. However, the degree of improvement in the treatment group was significantly greater than that of the control group. The improvement in symptoms in patients of the treatment group was sustained at follow-up, whereas that of the control group was not. The overall efficacy of the treatment was significantly greater than that of the control. Both groups demonstrated significant improvements in quality-of-life ratings after treatment, but the improvement was significantly greater in the treatment group than in the control group. In addition, the patients in the treatment group showed significantly increased HGB and significantly decreased

CRP levels and histopathological scores at the end of treatment, whereas the control group did not exhibit significant changes.

CONCLUSION: Moxibustion with acupuncture provided significant therapeutic benefits in patients with active CD beyond the placebo effect and is therefore an effective and safe treatment for active CD.

Core tip: Acupuncture treatment has been widely used in the clinical treatment of various diseases, particularly gastrointestinal diseases. Crohn's disease (CD) is a type of inflammatory bowel disease, and its incidence increases each year in China. However, there are limited numbers of reports on the efficacy of acupuncture treatment for CD. In the present study, we found that acupuncture provided significant therapeutic benefits to patients with mild to moderate CD and is therefore an effective and safe treatment.

INTRODUCTION

Crohn's disease (CD) is a type of inflammatory bowel disease and is a recurrent systemic inflammatory disease that mainly affects the gastrointestinal tract, with extraintestinal manifestations and related immune diseases[1]. With a steadily increasing incidence, CD has become a common digestive disease in Asia, especially in China[2]. CD has a significant impact on the productivity of society and on personal quality of life[3-6], and places a major burden on public health care resources[7]. Current medicinal treatments for CD primarily utilize salicylic acid-based drugs, corticosteroids, immunosuppressives, and biological agents. However, the poor efficacy and significant adverse effects of these treatments and the susceptibility to recurrence after dose reduction or discontinuation limit the long-term clinical application of these drugs[8].

Acupuncture has a history of more than 4000 years and is popular in China and many other countries. Acupuncture has been widely used for the clinical treatment of various diseases, particularly gastrointestinal diseases such as CD[9,10], ulcerative colitis[11,12], irritable bowel syndrome[13,14], and functional dyspepsia[15,16]. However, there are a limited number of reports on the clinical study of acupuncture in the treatment of CD[17], and its efficacy is therefore not fully established. In particular, the effects of acupuncture on endoscopic findings and the intestinal histopathological changes of CD have not been reported. Therefore, it is necessary to perform an objective evaluation of the efficacy of acupuncture in CD.

Our research team has performed clinical and basic research on acupuncture and moxibustion for the treatment of inflammatory bowel disease for over 30 years. Herb-partitioned moxibustion combined with acupuncture therapy is commonly used for the treatment of CD. Herb-partitioned moxibustion, a critical component of moxibustion therapy, is performed by placing a cake of herbs (dispensing a traditional Chinese medicinal (TCM) formula) on the patient's acupoints, followed by the placement and ignition of moxa cones, which are composed of refined mugwort floss, on the herbal cake to treat diseases. Acupuncture is a collection of procedures involving penetration of the skin with needles to stimulate certain points on the body. Here, we conducted a randomized controlled clinical trial of herb-partitioned moxibustion combined with acupuncture for the treatment of active CD with the goal of evaluating its clinical efficacy and safety.

MATERIALS AND METHODS

Study design

From January 2010 to April 2013, CD patients treated at the acupuncture outpatient center for inflammatory bowel disease of the Shanghai Institute of Acupuncture and Meridian, the Endoscopy Center of Zhongshan Hospital at Fudan University, the Department of Acupuncture-Moxibustion of Shuguang Hospital affiliated with the Shanghai University of Traditional Chinese Medicine, and the Yueyang Hospital of Integrated Traditional Chinese and Western Medicine affiliated with the Shanghai University of Traditional Chinese Medicine were recruited as subjects for this study. The diagnosis of CD was confirmed in all patients by clinical manifestation evaluation, imaging analysis, and endoscopic and histopathological examinations.

This clinical trial was approved by the Ethics Committee of the Yueyang Hospital of Integrated Chinese and Western Medicine affiliated with the Shanghai University of TCM. All subjects provided informed consent prior to enrollment into the trial. The study was registered in the Clinical Trials Registry at http://clinicaltrials. gov/(NCT01697761).

Patients

Patients who had a confirmed diagnosis of mild or moderate CD (CD Activity Index (CDAI) values ranging from 151 to 350), had not taken medications such as salicylic acid drugs and/or prednisone (at a dose $\leqslant$ 15 mg) for at least 1 mo, and had not taken immunosuppressants or used anti-TNF-α biological agents for 3 mo prior to

Table 1 Acupoints selected for the treatment and control groups

	Treatment group	Control group
Acupuncture points	Zusanli (ST36)	20 mm away from the posterior of ST36
	Shangjuxu (ST37)	20 mm away from the posterior of ST37
	Gongsun (SP4)	On the medial aspect of the 1st cuneiform bone, between LR4 and SP4
	Sanyinjiao (SP6)	15 mm away from the anterior of SP6, on the medial aspect of the tibia
	Taixi (KI3)	15 mm away from the anterior of KI3, on the medial aspect of the tibia
	Taichong (LR3)	On the dorsal aspect of the 1st metatarsal bone between LR3 and SP3
Moxibustion points	Tianshu (ST25), Qihai (CV6), Zhongwan (CV12)	

ST: Stomach; CV: Conception vessel; SP: Spleen; LR: Liver; KI: Kidney.

enrollment in the study were included.

Pregnant or lactating patients; patients with serious diseases of the heart, brain, liver, kidney, or hematopoietic system; patients with mental illness; and patients with other severe diseases were excluded.

After enrollment, the patients who were using CD medications maintained their drug dosage unchanged. If their conditions deteriorated during the treatment period or if the patients needed to increase their dose or take other medications, these subjects were withdrawn from the study. During the follow-up period, patients were allowed to adjust their dose of Western medicine after recording each adjustment. If patients increased their dose, became sicker, or took other drugs, these subjects were also withdrawn.

Randomization and blinding

We used a simple random sampling method by generating a random number table using the SPSS 16.0 software. The first number in the random number table was used as a starting point to create random assignment cards, which were then sealed in envelopes. The envelopes were numbered (the same number as the sequence number of the card inside) and kept secure by a dedicated person. When a qualified participant was enrolled into the trial, researchers then asked this dedicated person for a random number by phone or text message. According to the order in which the patient was enrolled, the person in charge opened the envelope with the same sequence number and informed the researchers of the assigned information by phone or text message. The odd random numbers were assigned to the treatment group, and even numbers were assigned to the control group. The participants were randomly divided into the treatment and control groups at a ratio of 1:1.

All patients were blinded during the trial and were therefore unaware of the specific treatment they received. All subjects in each treatment session were treated in a private room to avoid potential communication and comparison among subjects. In addition, a blinded evaluation was conducted in which a third researcher who was unaware of the group assignments assessed the treatment outcomes. Blinded statistical data analysis was also conducted in which the researchers, operators, and statisticians were separated from one another.

Interventions

The treatment group received herb-partitioned moxibustion combined with acupuncture. The acupoints are listed in Table 1 and Figure 1; these acupoints were selected based on TCM principles according to the clinical manifestations of the patients. All of these acupoints were shown to be effective for the treatment of CD in our previous report[10].

The herbal cake recipe used for moxibustion in the treatment group included *Coptis chinensis*, Radix *Aconiti Lateralis*, *Cortex Cinnamomi*, Radix Aucklandiae, *Flos Carthami*, *Salvia miltiorrhiza*, and *Angelica sinensis* as the main ingredients[10]. These herbs were ground into fine powders, which were then passed through a 100-mesh sieve and stored for future use. During treatment, 2.8 g of the herbal powder was mixed with maltose and made into a thick paste by adding warm water. The paste was then pressed into a mold to make herbal cakes with a diameter of 28 mm and a thickness of 5 mm. For moxibustion, pure refined moxa sticks ("Hanyi", Nanyang, China, size: 17 mm × 200 mm) were cut into segments of 16 mm in height that weighed approximately 1.8 g. In each session, each acupoint was treated with two segments of moxa sticks.

Positioning of the acupuncture points was based on the "Nomenclature and location of acupuncture points" (Chinese National Standard (GB/T12346-2006)). Sterile disposable stainless steel needles (with a diameter of 0.30 mm and length of 40 mm or 25 mm, "Huatuo", Suzhou, China) were used. The needles were directly inserted 20-30 mm into the skin and elicited a *de-qi* sensation. The needle was kept in position for 30 min. Herb-partitioned moxibustion and acupuncture were performed at the same time once every other day (three times per week) for a total of 36 sessions (12 wk). Subjects who received at least 80% of the required number of treatment sessions (29 or more) were considered to have completed the entire treatment plan.

The control group received wheat bran-partitioned moxibustion combined with superficial acupuncture. Wheat bran-partitioned moxibustion is often used by our research team as a placebo control method, and previous studies have shown that wheat bran-partitioned moxibustion differs significantly from herb-partitioned moxibustion[12]. The acupoints used in wheat bran-partitioned moxibustion were the same as those used for the treatment group. The procedure of superficial needling at non-acupoints was based on previous studies[15]. Non-meridian and non-acupoint zones located 1-2 cm away from the acupoints of the treatment group were selected for the control group, and an equal number of points
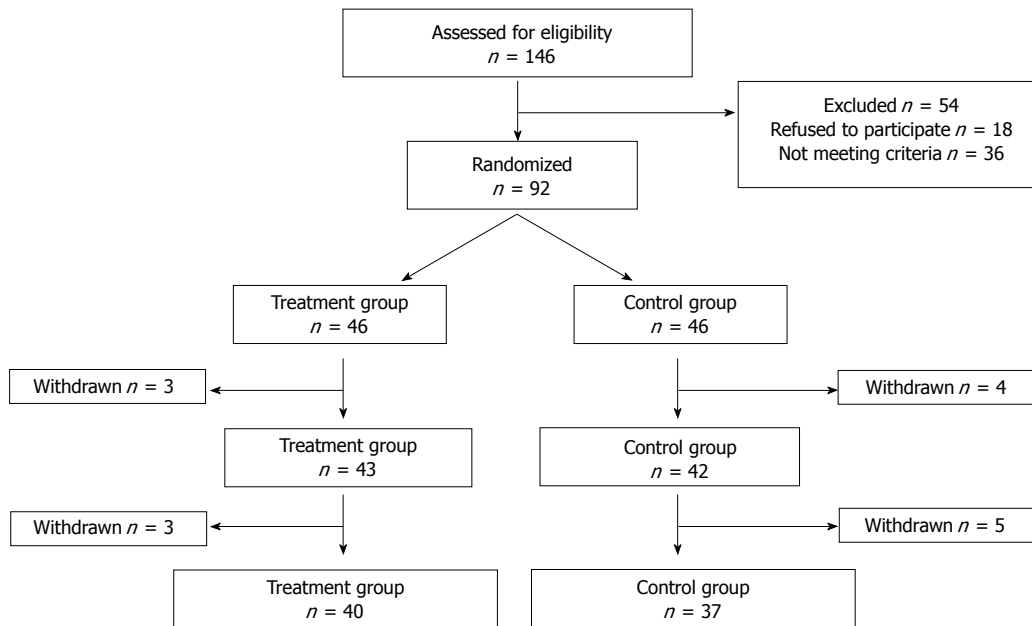
Figure 1 Flow chart of the trial.

were used in each group (Figure 1).

Wheat bran was used instead of herbs for the control group partition cake, and the treatment methods were the same as those of the treatment group. Needles with the same diameter and length as those used in the treatment group were used for needling; however, the needles were directly inserted only 1-2 mm into the skin, without eliciting a *de-qi* sensation. Wheat bran-partitioned moxibustion and acupuncture were performed at the same time, and the sessions were the same as those of the treatment group.

In this study, the acupuncture practitioners were all clinicians who were qualified TCM practitioners and had at least 2 years of clinical experience.

Outcome measures

The CDAI[18] was used as the main measurement of outcome. This index consists of eight factors, with each factor totaled after adjustment with a weighting factor. CDAI evaluations were performed on the day of enrollment, in the 12th week after enrollment, and at follow-up (24th week). The changes in CDAI score and total treatment efficacy were evaluated. The total treatment efficacy evaluation standards were as follows: after treatment, a CDAI score measuring less than 150 indicated clinical remission, a CDAI score decreased by more than 70 indicated an improvement, and a CDAI score decreased by less than 70 or an increased CDAI score indicated an ineffective treatment. The effective rate = (the number of patients who demonstrated clinical remission + the number of patients who demonstrated an improvement with treatment)/the total number of patients.

Six parameters were used as secondary outcome measures. The quality of life of the patients was evaluated using the inflammatory bowel disease questionnaire

(IBDQ), which is specific for patients with inflammatory bowel disease[19,20]. The level of C-reactive protein (CRP) and the erythrocyte sedimentation rate (ESR) were used to assess the level of intestinal inflammation, and hemoglobin (HGB) measurements were used to assess the presence and severity of anemia. Fifteen patients from each group were selected to receive enteroscopic examinations and mucosal biopsies. The CD endoscopic index of severity (CDEIS)[21] and the D'Haens-Geboes intestinal histology scoring system[22] were used to evaluate endoscopic mucosal manifestations and the pathomorphological and inflammatory conditions of the patients, respectively. Intestinal mucosa samples obtained from biopsy were immediately placed in 10% formalin for storage. After hematoxylin and eosin (HE) staining, the tissues were imaged, and the pathological changes were scored. All secondary outcomes were measured on the day of enrollment and during the 12th week after enrollment in the trial.

All patients were followed-up by phone, email, or other methods in the 24th week regarding changes in disease and medication conditions. During the follow-up period (weeks 12-24), patients who had aggravated symptoms with a CDAI score greater than 150 and a CDAI score increase greater than 70 points were defined as patients with recurrence.

The safety evaluation included the following three aspects: vital sign monitoring including post-treatment temperature, respiration, heart rate, blood pressure, and liver and kidney function; acupuncture abnormalities including bleeding, hematoma, fainting during acupuncture treatment, pain in the acupuncture sites, increased blood pressure, and other adverse reactions; and moxibustion abnormalities including skin burns, blistering, or other discomfort caused by moxibustion.
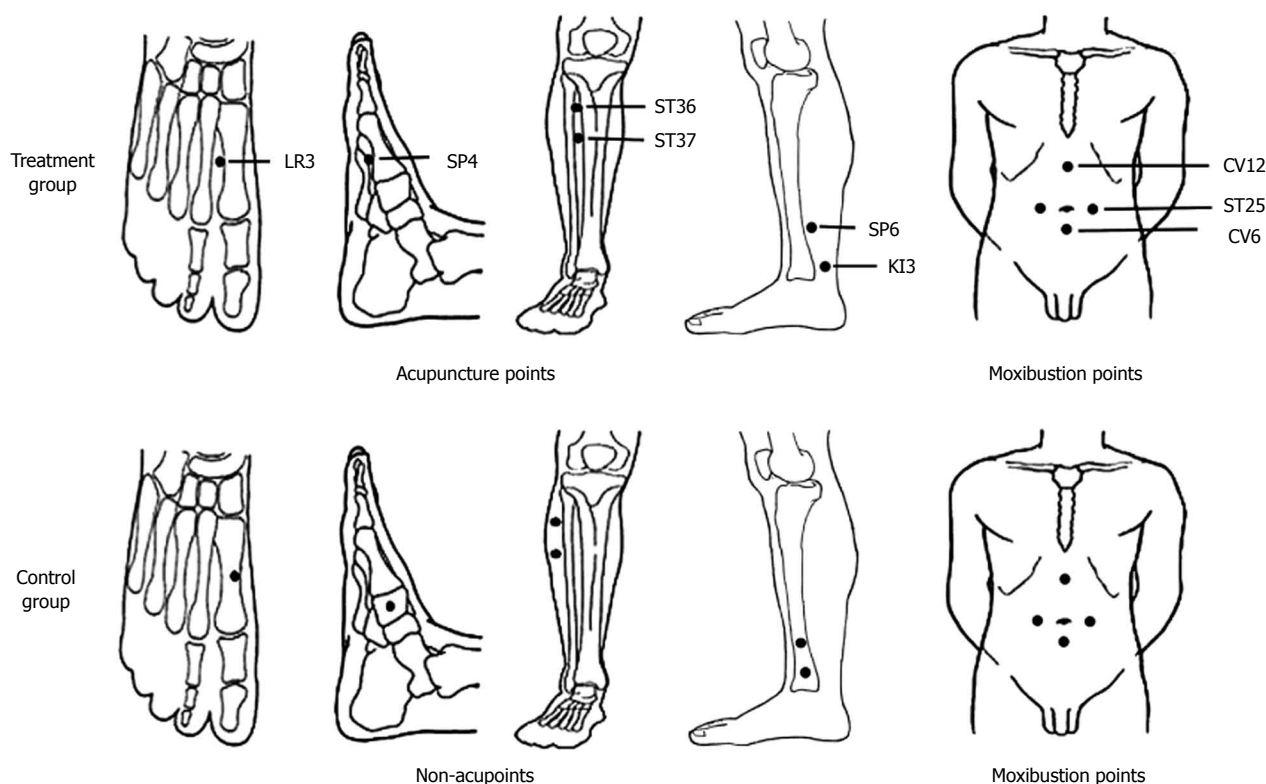
Figure 2 Locations of acupoints and non-acupoints used in this trial. ST: Stomach; CV: Conception vessel; SP: Spleen; LR: Liver; KI: Kidney.

Statistical analysis

According to the literature, the effective rate of acupuncture and moxibustion in the treatment of CD is 56%[9]. Our group previously used a non-randomized concurrent control method to conduct a pilot study that demonstrated an effective rate of herb-partitioned moxibustion and acupuncture in the treatment of CD of 86.67%[10]. Therefore, the current study established an expected effective rate value of 85%. The study consisted of two groups, with a significance level of $\alpha = 0.05$ (one-sided) and a test power of $1 - \beta = 0.9$. The formula for sample size estimation in comparing two sample rates was as follows:

$$n = (U_\alpha + U_\beta)^2 (1 + 1/k) \, P \, (1 - P)/(P_e - P_c)^2$$

Based on this calculation, the required sample size for each group was equal to 42 ($n = 42$ patients). With the addition of a 10% dropout rate (four patients), the two groups needed to include no less than 92 patients.

Statistical analyses of the baseline information and disease-related information were performed using the SPSS 16.0 software package (SPSS Inc., Chicago, IL, United States). The primary efficacy indicator, CDAI, was analyzed using per-protocol (PP) analysis and intention-to-treat (ITT) analysis, and the secondary efficacy indicator and subgroup analyses were performed using PP analysis. Normally distributed measurement data were analyzed using the *t*-test, and data that did not pass the normality test were analyzed using a non-parametric test (Mann-Whitney test). Count data were analyzed using the χ^2 test. All tests were two-sided, and $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Of 146 screened patients, 26 could not be included due to CDAI scores that were too low ($n = 22$) or too high ($n = 4$), refusal to participate ($n = 18$), or other reasons including the use of immunosuppressive therapy or traditional Chinese drugs ($n = 10$). A total of 92 patients were assigned to the treatment ($n = 46$) and control ($n = 46$) groups. A total of 85 patients completed the trial: of the patients in the treatment group, 43 completed the treatment, and three did not (two patients reported work obligations and one patient traveled abroad); of the patients in the control group, 42 completed the treatment and four did not (two patients presented deteriorating conditions and received prednisone at doses > 15 mg/d, one patient became pregnant, and one quit for unknown reasons). A total of 77 patients completed the follow-up study, including 40 in the treatment group and 37 in the control group (Figure 2).

There were no statistically significant differences between the treatment group and control group in baseline data including patient age, gender, height, weight, disease duration, surgical history, disease severity, CDAI, quality of life, and Montreal classification (age at diagnosis, lesion location, and lesion type) (Table 2).

CDAI scores

PP analysis demonstrated that the post-treatment CDAI scores of patients in the treatment group and the control group were significantly reduced compared with

Table 2 Patient characteristics at baseline

Characteristics	Treatment group	Control group	*P* value[1]
Age (yr); mean ± SD	36.98 ± 14.46	32.38 ± 11.83	0.113
Gender (male/female)	27/16	25/17	0.757
Height (cm)	167.47 ± 9.20	166.71 ± 6.79	0.670
Weight (kg)	53.69 ± 11.73	53.44 ± 11.73	0.924
Duration of disease (yr)	4.64 ± 3.75	4.67 ± 4.51	0.978
Prior resection (yes/no)	8/35	4/38	0.229
Severity (mild/moderate)	24/19	30/12	0.135
CDAI	210.84 ± 48.03	201.04 ± 57.13	0.394
IBDQ	157.67 ± 30.43	157.36 ± 33.19	0.963
HGB	118.05 ± 17.79	124.79 ± 20.70	0.111
ESR	21.80 ± 16.86	24.64 ± 22.28	0.625
CRP	18.52 ± 24.35	12.32 ± 13.43	0.164
Concomitant medication	27	24	0.398
Aminosalicylates	16	11	
Corticosteroids	2	3	
Aminosalicylates and corticosteroids	9	10	
Montreal classification			
Age at diagnosis			
A1	2	3	0.373
A2	32	33	
A3	9	6	
Location			
L1	6	8	0.251
L2	8	11	
L3	29	23	
L4	0	0	
Behavior			
B1	12	9	0.853
B2	2	2	
B3	10	12	
B1P	7	10	
B2P	3	1	
B3P	9	8	

[1]*P* values from comparisons between the treatment and control groups. CDAI: Crohn's disease activity index; IBDQ: Inflammatory bowel disease questionnaire; HGB: Hemoglobin; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein.

those measured at baseline (*P* = 0.000). Patients in the treatment group showed a significantly larger decrease in CDAI scores than the control group (*P* = 0.000). Furthermore, in the treatment group, the CDAI score during the follow-up period was significantly lower than the pretreatment baseline (*P* = 0.000). Although the CDAI score of the control group was decreased during the follow-up period, the change was not statistically significant (*P* = 0.056). However, the difference in CDAI scores during the follow-up period between the two groups was statistically significant (*P* = 0.000). In both groups, changes in CDAI scores between the follow-up period and the post-treatment time were not significant (treatment group *P* = 0.094, control group *P* = 0.064) (Table 3, Figure 3A).

The ITT analysis results were generally consistent with those of the PP analysis, with the exception that the ITT analysis showed a significant difference between the CDAI score of the control group during follow up and the baseline level before treatment (Table 3, Figure 3B).

Based on the principle that only a value equal to or greater than the minimal clinically important difference

(MCID) has clinical significance, the MCID was determined to be 70 points on the CDAI scale[23], demonstrating that changes in the treatment group were clinically significant both after treatment and in the follow-up period, whereas changes in the control group were not clinically significant after treatment or in the follow-up period.

Total efficacy evaluation

The PP analysis indicated that the total treatment efficacies of the treatment and control groups were 83.72% and 40.48%, respectively, with a statistically significant difference (*P* = 0.000). The ITT analysis indicated that the total treatment efficacies of the treatment and control groups were 78.26% and 36.96%, respectively, with a statistically significant difference (*P* = 0.000) (Table 4).

Quality-of-life surveys

All post-treatment IBDQ scores of patients in the treatment and control groups were increased from the pre-treatment baseline scores, with both groups showing statistically significant intragroup differences (*P* = 0.000). The patients in the treatment group showed a larger increase in IBDQ score than the patients in the control group, and this difference between the groups was statistically significant (*P* = 0.017) (Table 5).

Laboratory tests

Compared with the baseline values obtained prior to treatment, the HGB levels of the patients in the treatment group were significantly increased after treatment (*P* = 0.026), whereas those of the control group were not. Furthermore, the difference in HGB level between the two groups following treatment was statistically significant (*P* = 0.029) (Table 5).

Compared with the baseline values before treatment, the CRP levels of the patients in the treatment group were significantly reduced after treatment (*P* = 0.007). Moreover, the CRP levels of the control group did not change significantly, although the difference between the two groups was statistically significant (*P* = 0.008) (Table 5).

Compared with the baseline values before treatment, the ESR levels of the patients in the treatment and control groups were similarly decreased. However, there was no significant difference between the two groups (Table 5).

Colonoscopy evaluation

The treatment and control groups both showed reduced CDEIS scores following treatment when compared with pre-treatment scores; however, these differences were not statistically significant. Likewise, there was no significant difference between the two groups (Table 5).

Histopathological scores

Compared with pre-treatment assessment, the treatment and control groups both showed reduced histopathological scores following treatment. However, only the treatment group showed a statistically significant difference (*P*

Table 3 Results of the main outcome measurement: Crohn's disease activity index score (per-protocol and intention-to-treat analysis) (mean ± SD)

Analysis set	Group	*n*	Baseline	Changes from baseline to post-treatment	*P* value[1]	*n*	Changes from baseline to follow up	*P* value[1]
PP analysis	Treatment	43	210.84 ± 48.03	-115.35 ± 55.05[b]	0.000	40	-128.93 ± 64.46[b]	0.000
	Control	42	201.04 ± 57.13	-35.68 ± 46.91[b]		37	-14.32 ± 52.09	
ITT analysis	Treatment	46	211.81 ± 50.97	-107.83 ± 60.47[b]	0.000	46	-117.85 ± 70.10[b]	0.000
	Control	46	200.20 ± 54.91	-32.58 ± 45.91[b]		46	-22.19 ± 55.31[b]	

[1]*P* values from comparisons between the treatment and control groups. [b]*P* < 0.01 *vs* control group. PP: Per-protocol; ITT: Intention-to-treat.
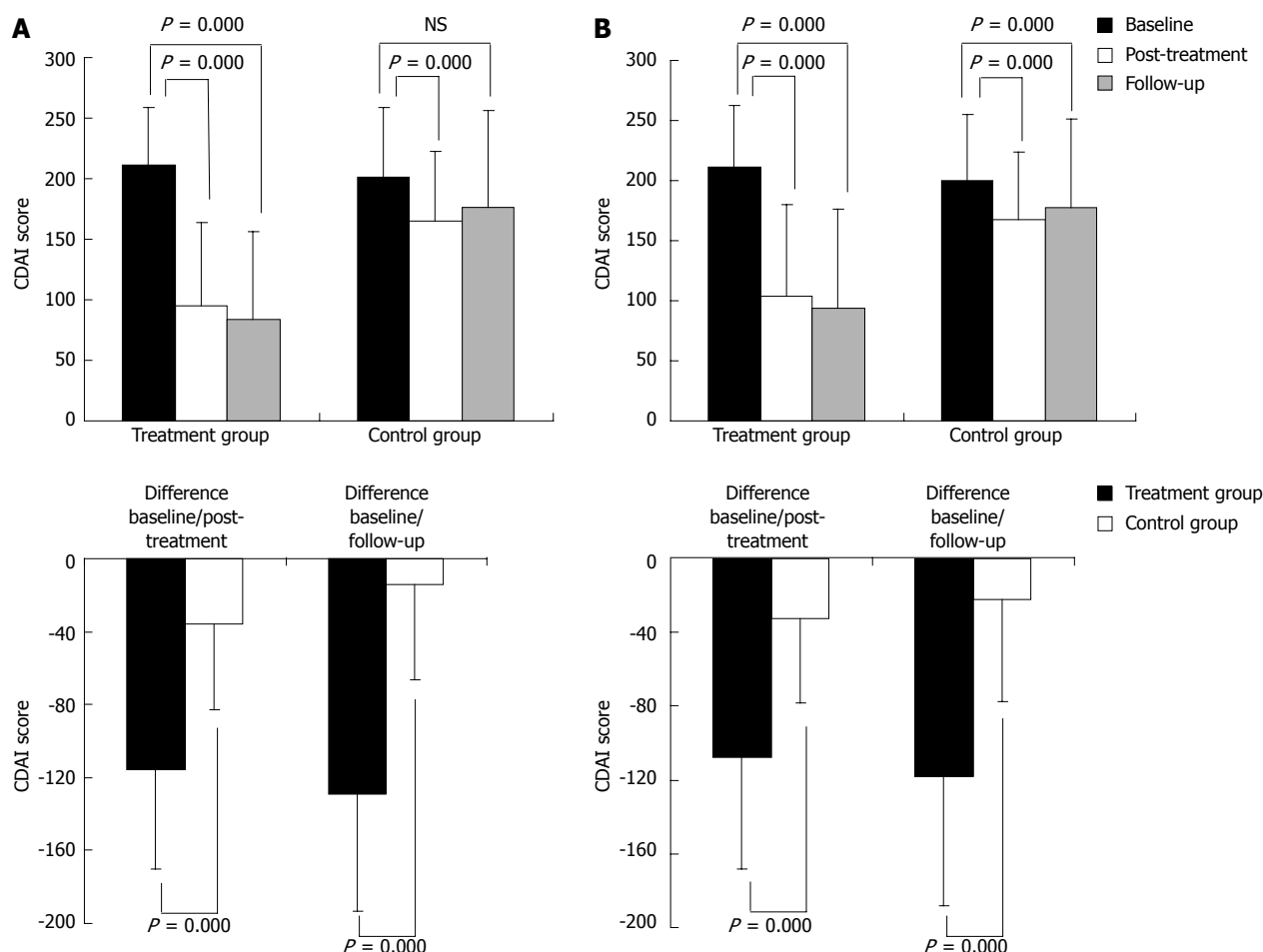
Figure 3 Main outcome measures (Crohn's disease activity index score). A: PP analysis; B: Intention-to-treat analysis; CDAI: Crohn's disease activity index.

= 0.002), and the difference between the two groups was also statistically significant (*P* = 0.029) (Table 5).

Subgroup analysis

We also performed subgroup analysis to assess changes in the CDAI score, which served as the primary efficacy indicator, between the two groups of patients after treatment based on the subcategories of gender (male/female), disease severity (mild/moderate), the use of corticosteroids, and lesion position classification (L1-Ileal; L2-Colonic; L3-Ileocolonic). The results showed that in all subcategories, patients in the treatment and control groups did not differ significantly in terms of pre-treatment baseline values and were thus comparable.

However, the subgroup analysis comparing the patient values after treatment with the pre-treatment values demonstrated that the patients in the treatment group differed significantly from the control group in terms of CDAI score changes in all subcategories, including gender (male/female), disease severity (mild/moderate), the use of corticosteroids, and lesion position classification (L1/L2/L3) (Table 6).

Follow-up period assessment

A total of 77 patients, including 40 patients in the treatment group and 37 patients in the control group, completed the follow-up period. Five patients (12.5%) in the treatment group relapsed, and 12 patients (32.4%) in the

Table 4 Results of the total efficacy evaluation (per-protocol and intention-to-treat analysis analysis)

Analysis set	Group	*n*	Clinical remission	Improved	Ineffective	*P* value
PP analysis	Treatment	43	32	4	7	0.000
	Control	42	15	2	25	
ITT analysis	Treatment	46	32	4	10	0.000
	Control	46	15	2	29	

P values from comparisons between the treatment and control groups. PP: Per-protocol; ITT: Intention-to-treat.

Table 5 Results of the secondary outcome measures (*n* = 85) (mean ± SD)

Outcome measures	Group	Baseline	Post-treatment	Changes from baseline to post-treatment	*P* value
IBDQ	Treatment	157.67 ± 30.43	182.23 ± 33.07[b]	24.56 ± 34.15	0.017
	Control	157.36 ± 33.19	167.29 ± 29.85[b]	9.93 ± 19.13	
HGB	Treatment	118.05 ± 17.79	123.14 ± 20.87[a]	5.09 ± 14.45	0.029
(g/L)	Control	124.79 ± 20.70	123.86 ± 21.23	-0.93 ± 10.07	
ESR	Treatment	21.80 ± 16.86	17.85 ± 14.14	-3.77 ± 13.00	0.163
(mm/h)	Control	24.64 ± 22.28	24.41 ± 22.10	-0.21 ± 10.12	
CRP	Treatment	18.52 ± 24.35	8.71 ± 8.94[b]	-8.67 ± 20.04	0.008
(mg/L)	Control	12.32 ± 13.43	13.60 ± 14.81	1.16 ± 12.11	
CDEIS	Treatment	9.92 ± 8.94	7.64 ± 6.84	-2.28 ± 5.52	0.380
	Control	6.15 ± 3.90	5.54 ± 4.79	-0.60 ± 4.75	
HS	Treatment	11.2 ± 1.47	9.00 ± 2.11[b]	-2.2 ± 2.21	0.029
	Control	11.07 ± 1.44	10.53 ± 2.13	-0.53 ± 1.73	

P values from comparisons of changes from baseline to post treatment between the treatment and control groups ([a]$P < 0.05$; [b]$P < 0.01$ *vs* control). IBDQ: Inflammatory bowel disease questionnaire; HGB: Hemoglobin; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; CDEIS: Crohn's disease endoscopic index of severity.

Table 6 Changes in Crohn's disease activity index scores according to subgroup (baseline to post treatment) (*n* = 85) (mean ± SD)

Subgroup (treatment group/control group)	Treatment group	Control group	*P* value[1]
Sex			
Male (27/25)	-118.84 ± 47.29	-48.97 ± 51.95	0.001
Female (16/17)	-109.48 ± 67.45	-16.14 ± 30.12	0.001
CDAI at baseline			
CDAI ≤ 220 (24/30)	-110.18 ± 49.51	-26.87 ± 43.11	0.001
CDAI > 220 (19/12)	-121.89 ± 62.10	-57.70 ± 50.59	0.005
Treatment with corticosteroids			
Yes (11/13)	-131.30 ± 34.25	-28.86 ± 32.35	0.001
No (32/29)	-109.87 ± 60.05	-38.73 ± 52.36	0.001
Location			
L1-Ileal (6/8)	-96.77 ± 55.42	-19.70 ± 51.42	0.020
L2-Colonic (8/11)	-114.31 ± 34.93	-50.29 ± 46.47	0.005
L3-Ileocolonic (29/23)	-119.49 ± 60.00	-34.25 ± 45.56	0.001

[1]*P* values from comparisons between treatment and control groups. CDAI: Crohn's disease activity index.

control group relapsed. This difference between the two groups was statistically significant ($P = 0.035$).

Safety measures

A total of two adverse events were reported (2/92) during this clinical trial: one patient in the treatment group experienced pain or subcutaneous hematoma during acupuncture, and one patient in the control group received a mild burn during moxibustion. There were no serious adverse events.

DISCUSSION

This randomized controlled study evaluated the treatment efficacy of acupuncture (herb-partitioned moxibustion combined with acupuncture) for the treatment of mild to moderate CD, using placebo acupuncture (wheat bran-partitioned moxibustion combined with superficial acupuncture) as the control. The results showed that both acupuncture and placebo acupuncture significantly reduced the CDAI score of CD patients and improved

their quality of life. In addition, the efficacy of acupuncture was significantly superior to placebo acupuncture, indicating that herb-partitioned moxibustion combined with acupuncture has significant therapeutic effects in addition to the placebo effect.

After 12 wk of treatment, 74% of patients in the treatment group and 36% of patients in the control group entered remission (CDAI score < 150). After treatment, 70% of patients in the treatment group and 14% of patients in the control group showed CDAI score reductions greater than 100 points. In addition, 79% of patients in the treatment group and 21% of the control group patients demonstrated a CDAI score reduction greater than 70 points. ITT analysis of follow-up observations showed that both acupuncture and placebo acupuncture had fairly good long-term efficacies and significantly reduced CDAI scores compared with the baseline level prior to treatment. Additionally, these results revealed that the conditions of the patients were maintained at a level comparable to that immediately following treatment. The PP analysis results were similar to those of the ITT analysis, except that the PP analysis showed no significant differences in the CDAI scores of the control group measured at follow-up *vs* baseline, suggesting that the long-term efficacy of placebo acupuncture might not be stable. Joos *et al*[9] also demonstrated that both acupuncture and placebo acupuncture reduced the CDAI score of patients with mild to moderate CD and that the effect of acupuncture was significantly superior to placebo acupuncture. In addition, follow-up observations indicated that acupuncture had more stable long-term efficacy, as the placebo acupuncture group demonstrated no significant difference compared with observations recorded before treatment, consistent with the results of our study. The above results suggest that acupuncture has stable short-term and long-term effects on controlling the degree of disease activity in patients with mild to moderate CD and that these effects are clearly advantageous in comparison with placebo acupuncture.

Subgroup analysis further confirmed that acupuncture treatment was significantly more effective than placebo acupuncture in the four subgroups of gender, disease severity (mild/moderate), the use of corticosteroids, and lesion position (L1/L2/L3), suggesting that acupuncture has a broad application scope for the treatment of CD and fairly good clinical efficacy for patients in all subcategories. The results of Joos *et al*[9] are generally consistent with our results, with the exception of a subgroup analysis based on CDAI score. In their study, the results were negative for patients with CDAI scores < 250 and positive for patients with CDAI scores ≥ 250. In contrast, we used 220 points as the cut-off score, as this score is commonly used to classify the severity of the disease as mild or moderate. It is possible that the negative results detected in the study by Joos *et al*[9] may be due to this different classification.

In addition, the study by Joos *et al*[9] also showed that acupuncture and placebo acupuncture significantly improved patient quality of life; however, there was no significant difference between the two groups, which may have been caused by the small sample size (fewer than 60 cases) and a shorter treatment course (4 wk). In addition, different acupuncture techniques might explain why the results differ between the two studies. The present study used herb-partitioned moxibustion combined with acupuncture, whereas the study by Joos *et al*[9] mainly applied acupuncture with needles, and only patients exhibiting yang deficiency and chill symptoms were supplemented with moxibustion treatment. Despite the fact that both studies used acupuncture combined with moxibustion for CD treatment, the differences in moxibustion methods and acupuncture points likely resulted in different treatment effects. Anemia is a common clinical manifestation in patients with CD. We chose HGB level to reflect the impact of acupuncture on peripheral red blood cell volume in patients with CD. Our results showed that acupuncture significantly increased the HGB level of CD patients, whereas placebo acupuncture had no such effects. In addition, during the active stage, CD patients have elevated CRP and ESR levels, and elevated CRP levels, in particular, often directly reflect the degree of bowel inflammatory activity[24,25]. Our results showed that acupuncture significantly reduced CRP levels in patients with CD, whereas placebo acupuncture had no such effects. The ESR levels of patients in the treatment group showed a downward trend after treatment compared with the baseline levels before treatment, but this difference was not statistically significant. In contrast, the ESR levels in patients of the control group did not change significantly. The above results suggest that acupuncture effectively controls the inflammatory response and eases intestinal inflammation. The study by Joos *et al*[9] also showed that CRP levels were reduced after acupuncture treatment, but their observed difference was not statistically significant.

We also assessed the effects of acupuncture on endoscopic findings and intestinal tissue histopathological scores in patients with CD. Neither the treatment group nor the control group showed significant changes in endoscopic findings after treatment, and longer observations may be required to observe any improvements in mucosal integrity. However, the patients in the treatment group showed significant reductions in intestinal tissue histology scores after treatment, and the difference between the treatment and control groups was statistically significant. The above results suggest that acupuncture may have certain beneficial effects on improving intestinal inflammation.

TCM theory suggests that weak spleen and dominant dampness are the common pathogenic mechanisms of CD patients, who exhibit varying degrees of kidney weakness and liver stagnation. Therefore, this study mainly utilized spleen enhancement and dampness reduction supplemented with kidney augmentation, soothing the liver, and qi regulation as the main treatment principle. Herb-partitioned moxibustion is a treatment method

that has been used for many years by our research team to treat diseases such as CD[10,26-28], ulcerative colitis[12,29-31], and irritable bowel syndrome[32,33]. The TCM ingredients in herbal cakes gently augment the spleen and kidney, remove dampness, and regulate qi. Thermal stimulation generated during moxa stick combustion strengthens the action of this effect. The acupuncture points used in the current treatment were based on the principles of Chinese medicine and the common pathogenesis of patients with CD. The control group in this study received wheat bran-partitioned moxibustion combined with superficial acupuncture to maintain patient blinding. In addition, the same acupoints were used in both wheat bran-partitioned moxibustion and herb-partitioned moxibustion treatments. Because the abdomen contains a relatively large number of meridians and acupoints, the local warm sensation produced by moxibustion often affects a large area of the abdomen. Therefore, although we chose a non-meridian and non-acupoint zone 1-2 cm away for the placebo group, the warm sensation likely spread to nearby acupoints, which may have been detrimental to the placebo moxibustion control. In addition, our group previously performed clinical studies on herb-partitioned moxibustion for the treatment of ulcerative colitis[12], using wheat bran-partitioned separated moxibustion as the placebo-control and the same acupoints for the two groups. The results showed that there were significant differences in the clinical efficacy between the herb-partitioned moxibustion treatment and the wheat bran-partitioned moxibustion treatment, suggesting that wheat bran-partitioned moxibustion is a fairly appropriate model for placebo moxibustion. Although both treatment methods produce thermal stimulation, different ingredients in the herbal cakes are the reasons underlying the differences in efficacy between the two groups. Moreover, we cannot rule out the effect of superficial acupuncture in the control group; this may be an important factor contributing to the effects produced in the control group in addition to the placebo effect. Although there is controversy regarding the use of superficial acupuncture as a placebo control because superficial acupuncture may produce treatment effects[34], a large number of studies have used this method[9,11,35-38]. Our study demonstrated positive safety features and high patient compliance; only two patients experienced mild adverse events, which can likely be avoided in future studies. In addition, acupuncture is highly cost-effective, representing a clear advantage in comparison with other medications for the treatment of CD[39-41].

Although our study and studies from other groups have shown that acupuncture has a positive effect as a treatment for CD, the mechanism of how acupuncture achieves its treatment efficacy is not fully understood. Our team previously conducted studies on the mechanisms of acupuncture for the treatment of CD. The results suggested that acupuncture and/or moxibustion might inhibit the abnormal expression of the inflammatory cytokines, TNF-α and TNFR1, in the colonic mucosa and peripheral blood of a rat model of CD. These changes may subsequently reduce colonic epithelial cell apoptosis, improve colonic epithelial barrier structure and function, and increase the expression of the colonic epithelial tight junction proteins occludin, claudin-1, and ZO-1 *via* the TNF-α/TNFR1 pathway, thereby reducing intestinal inflammation in CD model rats, restoring/protecting the colonic epithelial barrier, and ultimately achieving the goal of alleviating chronic bowel inflammation in CD[26-28].

The present study did have certain limitations. During the follow-up period, only the primary efficacy indicator, CDAI, was evaluated; the secondary efficacy indicators, such as IBDQ and laboratory parameters, were not recorded. Moreover, the patients' anxiety and depression scale scores were not measured in the present study. Thus, future trials should consider the above limitations.

In summary, the clinical efficacy of herb-partitioned moxibustion combined with acupuncture in mild to moderate CD was significantly better than placebo acupuncture. In addition to the placebo effect, the treatment provided significant additional therapeutic effects. Thus, herb-partitioned moxibustion combined with acupuncture represents an effective and safe therapy for the clinical treatment of mild to moderate CD.

COMMENTS

Background
In Asia, particularly in China, the incidence of Crohn's disease (CD) has increased steadily. Although certain medicinal treatments have been administered for CD, several disadvantages have limited their long-term clinical application. Therefore, increasing numbers of patients are seeking alternative medical therapies, particularly those involving acupuncture. However, the efficacy of acupuncture in the treatment of CD has not been fully established.

Research frontiers
This research team has performed clinical and basic research on acupuncture and moxibustion for the treatment of inflammatory bowel diseases, including CD and ulcerative colitis, for more than 30 years. Although previous studies have demonstrated that this treatment can relieve the symptoms of CD, more observation is required to confirm its efficacy on peripheral inflammation levels, the capacity of blood cells, and intestinal endoscopic and histopathologic changes.

Innovations and breakthroughs
Herb-partitioned moxibustion and acupuncture not only reduced the disease activity index and improved quality of life, but also alleviated intestinal inflammation and improved hemoglobin levels in CD patients. Most importantly, few side effects were observed. The authors found that herb-partitioned moxibustion combined with acupuncture is an effective and safe treatment for mild to moderate CD. In addition to the placebo effect, it also provided significant therapeutic effects.

Applications
The results of the present study suggest that herb-partitioned moxibustion combined with acupuncture has the potential to be a very useful adjunct therapy for mild to moderate CD.

Terminology
Herb-partitioned moxibustion is a critical component of moxibustion therapy for disease treatment. It is performed by placing a cake of herbs (dispensing a traditional Chinese medicinal formula) on the patient's acupoints, followed by the placement and ignition of moxa cones, composed of refined mugwort floss, on the herbal cake.

Peer review
This is a well-conducted study that evaluates the clinical efficacy and safety of herb-partitioned moxibustion combined with acupuncture for the treatment of active CD. The authors show the overall efficacy of the treatment was significantly greater than that of the control. In addition, the patients in the treatment group showed significantly increased hemoglobin and significantly decreased

C-reactive protein levels and histopathological scores at the end of treatment, whereas the control group did not exhibit significant changes. Herb-partitioned moxibustion combined with acupuncture is therefore an effective and safe treatment method for mild and moderate CD.

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RANDOMIZED CONTROLLED TRIAL

Muscovite is protective against non-steroidal anti-inflammatory drug-induced small bowel injury

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Abstract

AIM: To evaluate the effect of muscovite in preventing small bowel injury induced by nonsteroidal anti-inflammatory drugs (NSAIDs).

METHODS: We recruited and screened thirty-two healthy volunteers who were randomly allocated equally into two groups: an NSAID control group, who received 75 mg slow-release diclofenac, twice daily for 14 d; and an NSAID-muscovite group, who received 3 g of muscovite in addition to the 75 mg of slow-release diclofenac, twice daily for 14 d. For gastroprotection, both groups were administered 20 mg/d of the proton pump inhibitor omeprazole. All eligible subjects underwent video capsule endoscopy (CE) prior to and 14 d after treatment.

RESULTS: Thirty subjects (NSAID-muscovite group, *n* =16; NSAID control group, *n* =14) finally completed the whole trail. At the baseline CE examination, no statistically significant differences between the two groups have been observed. However, after 14 d of drug treatment, a significant difference was observed in the percentage of subjects with mucosal breaks when comparing the NSAID-muscovite group with the NSAID control group. While 71.4% (10/14) of subjects in the NSAID control group had at least one mucosal break, co-administration of muscovite in the NSAID-muscovite group reduced the rate to 31.3% (5/16) (P = 0.028). Moreover, higher number of mucosal breaks was found in the NSAID control group *vs* that in the NSAID-muscovite group (P < 0.05).

CONCLUSION: Muscovite co-therapy reduced the incidence of small intestinal injury after 14 d of diclofenac administration.

Key words: Muscovite; Nonsteroidal anti-inflammatory drugs; Small intestinal injury; Video capsule endoscopy

Core tip: This is a randomized, open-label, controlled clinical trial to evaluate the incidence of small bowel damage by capsule endoscopy in healthy participants who received treatment with the nonsteroidal anti-inflammatory drug (NSAID) diclofenac and the effect of muscovite in preventing NSAID-induced small bowel injury.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs), one of the most commonly used classes of drugs worldwide, are widely accepted for their anti-inflammatory and analgesic properties. Although NSAIDs are beneficial in reducing pain and inflammation, they are also known to have adverse gastrointestinal (GI) effects. As cyclooxygenase-2 inhibitors were associated with an increased risk of cardiovascular events, conventional NSAIDs are more frequently prescribed by clinicians[1]. After the introduction of capsule endoscopy (CE) and due to the increased use of aspirin and NSAIDs, NSAID-induced gastric and duodenal mucosa damage has gained more attention. CE now allows for a full investigation and visualization of the entire small intestine. CE in patients has revealed that NSAID-induced lower GI injury is more common than NSAID-associated gastropathy[2-9]. The same CE studies showed that in up to 55% of healthy volunteers, co-administration of proton pump inhibitors (PPIs) with NSAIDs failed to prevent NSAID-induced small intestinal damage[10]. Co-administration of NSAIDs and misoprostol, a mucosal protective agent for the management of gastric ulcers, could attenuate mucosal damage, though this study lacked a large clinical sample[11]. Medications that prevent or treat NSAID-induced intestinal injuries are not currently available. It is critical to further understand the small intestinal damage induced by NSAIDs, because all clinicians, particularly gastroenterologists, should have a comprehensive understanding of the gastrointestinal adverse effects associated with NSAIDs.

Muscovite, a kind of natural clay or traditional Chinese medicine, is composed of insoluble double silicate of aluminum and magnesium. It has served in the management of gastric diseases in China for many years. Previous research from our lab has demonstrated that muscovite can reduce intestinal permeability in rats with NSAID-induced enteropathy. Moreover, muscovite also provides a protective effect against acute and sub-acute injuries of the intestinal mucosa[12,13]. The aim of the current study was to evaluate the effect of intragastric muscovite administration on intestinal injury induced by diclofenac treatment in healthy volunteers. This two-week, single-center study was a prospective, single-blinded, randomized, controlled study that utilized CE to evaluate the incidence of small bowel damage induced by NSAIDs in healthy subjects undergoing concomitant therapy with muscovite or not.

MATERIALS AND METHODS

Study subjects

From December 2012 through June 2013, we recruited and screened 32 healthy volunteers by CE and laboratory tests. Subjects that met the following criteria were eligible for inclusion in our study: no history of surgery, between the ages of 18 and 70 years, not taking any medication during the month prior to enrolment, and no abnormal findings from physical examinations or laboratory tests. All the subjects received a CE examination before enrolment. Subjects were excluded for the following reasons: (1) failure to traverse the full length of the small intestine; (2) the presence of stenosis, tumors, or ulcers; and (3) the number of mucosal breaks in the small intestine more than 5. Subjects with active gastrointestinal disease, ulcer and bleeding history, fecal occult blood test (+) or hemoglobin levels < 12 g/dL were further excluded from this study. This study was approved by the ethics committee of the First Affiliated Hospital of Zhejiang Chinese Medical University. Informed consent was obtained from each subject enrolled in this study before undergoing baseline CE examination.

Study protocol

All the eligible subjects were randomly allocated equally into two groups using a sorted random number generator. The subjects in the control group received 75 mg of diclofenac twice per day for 14 d, while the experimental group was co-administered the same dosage of diclofenac along with 3 g of muscovite twice daily for 14 d. Both groups were also given 20 mg of omeprazole daily for gastroprotection. All eligible subjects underwent CE prior to and 14 d after treatment. Post-treatment CE was conducted within 24 h after treatment was completed. Participants who discontinued treatment due to adverse effects or had incomplete post-treatment CE examination were also excluded.

CE

We used the OMOM video capsule system (Jinshan Science and Technology, Chongqing, China) in the current study. The CE procedures and methodology for image review were performed according to the study by Li *et al*[14]. After a 12-h fast, the subjects were requested to drink 50% magnesium sulfate 50 mL and 40 mg/mL simethicone 30 mL, respectively, 10 h and 15 min before the CE examination. All the participants were provided with recorder-battery belt pack and a sensor array. The capsule was swallowed with a cup of warm water, and two images were taken per second within 8 h. All the frames are transmitted continuous video images, and processed after unloading onto a computer. Following the preliminary CE examination, we briefly analyzed the results to determine whether participants were eligible for the further study. Two skilled technical reviewers independently screened per video for GI pathology, and the detected pathologies were further evaluated by two endoscopists who were blinded to the exact treatment protocol as well as participant characteristics. We saved all the images for a thorough analysis when all post-treatment CE examinations were accomplished.

Evaluation

The primary end point was the mean number of small intestinal mucosal breaks per subject. Table 1 describes the definition of any mucosal breaks as categories 1-5. The secondary end points included (1) the percentage of participants with at least one mucosal break of the small

Table 1 Assessment of small bowel lesions

0	Normal
1	Petechiae
2	Erosion
3	Ulcer (< 3)
4	Ulcer (≥ 3)
5	Other: denuded mucosa and lymphangiectasis

There were 6 categories of small bowel lesions. Each lesion was evaluated and assigned a category. Mucosal breaks included any lesion that appeared as an erosion or ulcer, regardless of perceived size.

Table 2 Characteristics of the subjects undergoing capsule endoscopy

Variable	NSAID-muscovite group	NSAID control group	*P* value
No. of subjects	16	14	NS[1]
Age (yr) (mean ± SD)	35.19 ± 15.86	33.50 ± 12.83	NS[1]
Sex (M/F)	6/10	7/7	NS[1]
Height (cm)	163.63 ± 7.92	165.71 ± 8.55	NS[1]
Body weight (kg)	58.63 ± 7.61	57.29 ± 9.91	NS[1]
No. of mucosal breaks	0.5 ± 1.4	0	NS[1]

[1]Calculated using the Student's *t* test; [1]Calculated using the Pearson χ^2 test. NSAID: Nonsteroidal anti-inflammatory drug; NS: Not significant. M: Male; F: Female.

bowel; (2) the severity of injury (categories 0-4 in Table 1); and (3) the type of injury (categories 1-5 in Table 1) in the small intestine. A post-hoc analysis was used to analyze the distribution of small intestinal mucosal breaks across intestinal tertiles. To do this, we grouped three equal areas between the cecum and duodenum according to the small bowel transit time of each participant. The participants were excluded from this particular specific analysis when the cecum was not clearly identified. Safety was assessed according to physical and laboratory findings, or observed and self-reported side-effects.

Statistical analysis
Age, sex, height, body weight and the number of mucosal breaks at baseline CE between the experiment and the control groups were analyzed by the Student's *t*-test. The percentage of subjects with at least one mucosal break between the two groups was analyzed by the Pearson χ^2 test. The injury severity and mean number of mucosal breaks per subject between the experiment and the control groups were evaluated by the Wilcoxon signed rank test. Data are presented as mean ± standard deviation (SD) if the values were normally distributed. $P < 0.05$ was set as the threshold for statistical significance.

RESULTS
Analysis of subjects
A flow chart depicting the study organization is presented in Figure 1. Thirty-two subjects underwent a baseline CE. Of the initial 32 participants, the entire small intes-
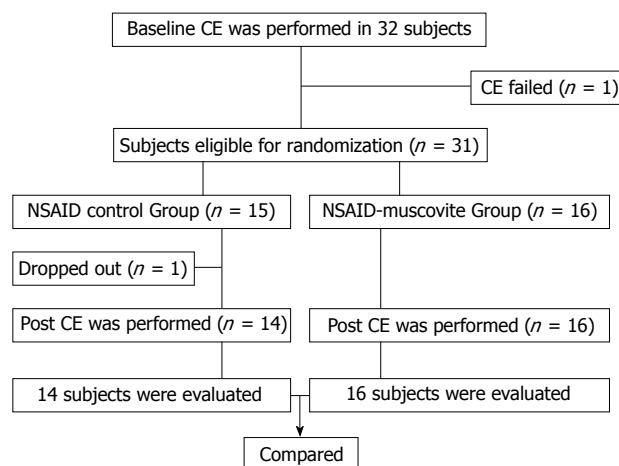

Figure 1 Flow chart depicting the study organization. NSAID: Nonsteroidal anti-inflammatory drug; CE: Capsule endoscopy.

tine was unable to observe in one participant, who was excluded from this study. There was no significant GI pathology in the remaining 31 participants, and they thus entered the study. Eligible subjects were then randomly assigned to either the NSAID control group or the NSAID-muscovite group. In the NSAID control group, one participant withdrew for personal reasons, and the remaining 14 participants accomplished the final study. All the 16 participants completed their therapy regimens in the NSAID-muscovite group. Thus, 14 participants in the NSAID control group and 16 subjects in the NSAID-muscovite group were finally evaluated for the presence of any mucosal break of the small bowel.

Baseline CE
The basic characteristics of each participant are shown in Table 2. There were no statistically significant differences in the baseline characteristics between the two groups during the initial CE examination. We observed 7 mucosal breaks in 2 of 16 participants (number of mucosal breaks: 0.5 ± 1.4) in the NSAID-muscovite group during the initial CE examination. No mucosal breaks were identified in the NSAID control group.

Post-treatment CE
After 14 d of treatment, the percentage of participants with at least one mucosal break of the small bowel was significantly higher in the NSAID control group [71.4% (10/14) of subjects] than in the NSAID-muscovite group [31.3% (5/16) of subjects] at the post-treatment CE ($P = 0.028$) (Figure 2). No statistically significant difference in the incidence of mucosal breaks was observed (12.5% before treatment and 31.3% after treatment; $P = 1.00$) in the NSAID-muscovite group (Table 3). We next analyzed the mean number of mucosal breaks in the participants who developed one or more mucosal breaks. The mean number of mucosal breaks in each participant increased in response to NSAID treatment in the NSAID control group; there were zero mucosal breaks at the baseline
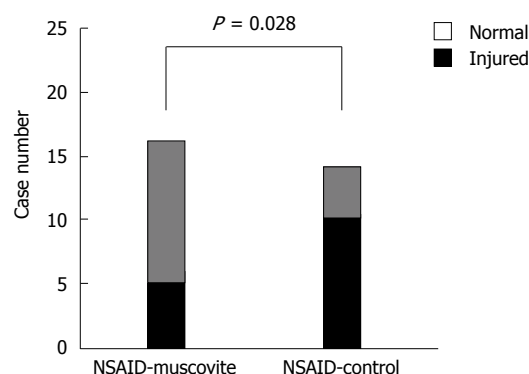
Figure 2 Percentage of subjects with at least one mucosal break at post-treatment capsule endoscopy. *P*-value was calculated by the χ^2 test. NSAID: Nonsteroidal anti-inflammatory drug.
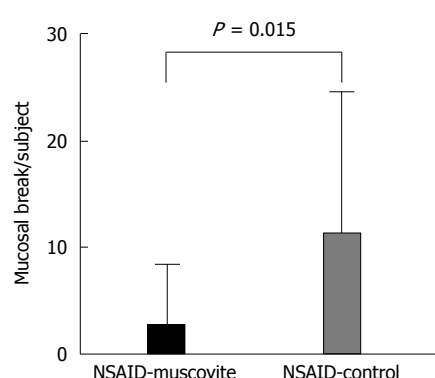

Figure 3 Mean mucosal breaks per subject at post-treatment capsule endoscopy. *P* value was calculated by the Wilcoxon signed rank test. NSAID: Nonsteroidal anti-inflammatory drug.

CE and 11.1 ± 13.5 at the end of treatment (*P* = 0.005). While there was no significant change in the number of mucosal breaks found (0.5 ± 1.4 before treatment and 2.5 ± 5.7 after treatment; *P* = 0.270) in the NSAID-muscovite group. Therefore, the mean number of mucosal breaks in each participant was increased in the NSAID control group *vs* the NSAID-muscovite group at the post-treatment CE examination (*P* = 0.015) (Figure 3, Table 4).

In the NSAID control group, we observed 28 (2.0 ± 3.0 per subject) episodes of petechiae in 7/14 subjects, 47 (3.4 ± 4.1 per subject) erosions in 10/14 subjects and 80 (5.7 ± 9.8 per subject) ulcers in 8/14 subjects after two weeks of NSAID administration. Treatment with muscovite reduced the incidence of mucosal breakdown to 14 (0.9 ± 2.5 per subject), episodes of petechiae in 3/16 subjects, 14 (0.9 ± 2.1 per subject) erosions in 4/16 subjects, and 12 (0.8 ± 2.0 per subject) ulcers in 4/16 subjects in the NSAID-muscovite group (Table 5). Representative examples of mucosal breaks observed in this study are shown in Figure 4.

We divided mucosal break severity into five levels (levels 0-4; Table 1): level 0: normal; level 1: petechiae; level 2: erosion; level 3: less than three ulcers; level 4: three or more ulcers observed. If the subjects had more than one type of mucosal break, we scored them at the highest

Table 3 Percentage of subjects with mucosal breaks at baseline and post-treatment capsule endoscopy *n* (%)

	Baseline	Post-treatment	*P* value[1]
NSAID-muscovite group	2 (12.5)	5 (31.3)	1
NSAID control group	0.000	10 (71.4)	-
P value[1]	0.485	0.028	

[1]Calculated using the Pearson χ^2 test. NSAID: nonsteroidal anti-inflammatory drug.

Table 4 Number of small bowel mucosal breaks in subjects with at least one break at baseline and post-treatment capsule endoscopy

	Baseline	Post-treatment	*P* value[1]
NSAID-muscovite group	0.5 ± 1.4	2.5 ± 5.7	0.270
NSAID-control group	0.000	11.1 ± 13.5	0.005
P value[1]	0.178	0.015	

[1]Calculated using the Wilcoxon signed rank test. NSAID: Nonsteroidal anti-inflammatory drug.

Table 5 Comparison of injuries observed in the nonsteroidal anti-inflammatory drug control group *vs* the nonsteroidal anti-inflammatory drug-muscovite group *n* (%)

Type of injury	NSAID-muscovite group	NSAID control group	*P* value[1]
Petechiae	3 (19)	7 (50)	0.070
Erosion	4 (25)	10 (71)	0.011
Ulcer	4 (25)	8 (57)	0.073
Denuded areas	1 (6)	3 (21)	0.315
Lymphangiectasis	1 (6)	8 (57)	0.004

[1]Calculated using the χ^2 test. NSAID: nonsteroidal anti-inflammatory drug.

Table 6 Classification of small bowel injury severity after treatment *n* (%)

Severity classification[1]	0	1	2	3	4	*P* value[2]
NSAID-muscovite group	11 (69)	0	1 (6)	3 (19)	1 (6)	0.017
NSAID control group	4 (27)	0	2 (14)	2 (14)	6 (43)	

[1]Severity classification: 0 Normal; 1 Petechiae; 2 Erosion; 3 Ulcer (< 3); 4 Ulcer (≥ 3); [2]Calculated using the Wilcoxon signed rank test. NSAID: Nonsteroidal anti-inflammatory drug.

level. In the NSAID control group, 57% (8/14) of the subjects had ulcers, with 43% (6/14) having at least three or more ulcers. In contrast, only 6% (1/16) of subjects in the NSAID-muscovite group had three or more ulcers. Thus, the severity of mucosal breaks observed in the NSAID control group was significantly greater compared with the NSAID-muscovite group (*P* = 0.017) at the post-treatment CE (Table 6).

Post-hoc analysis
We performed a post-hoc analysis and the distribution of participants with at least mucosal breaks is listed
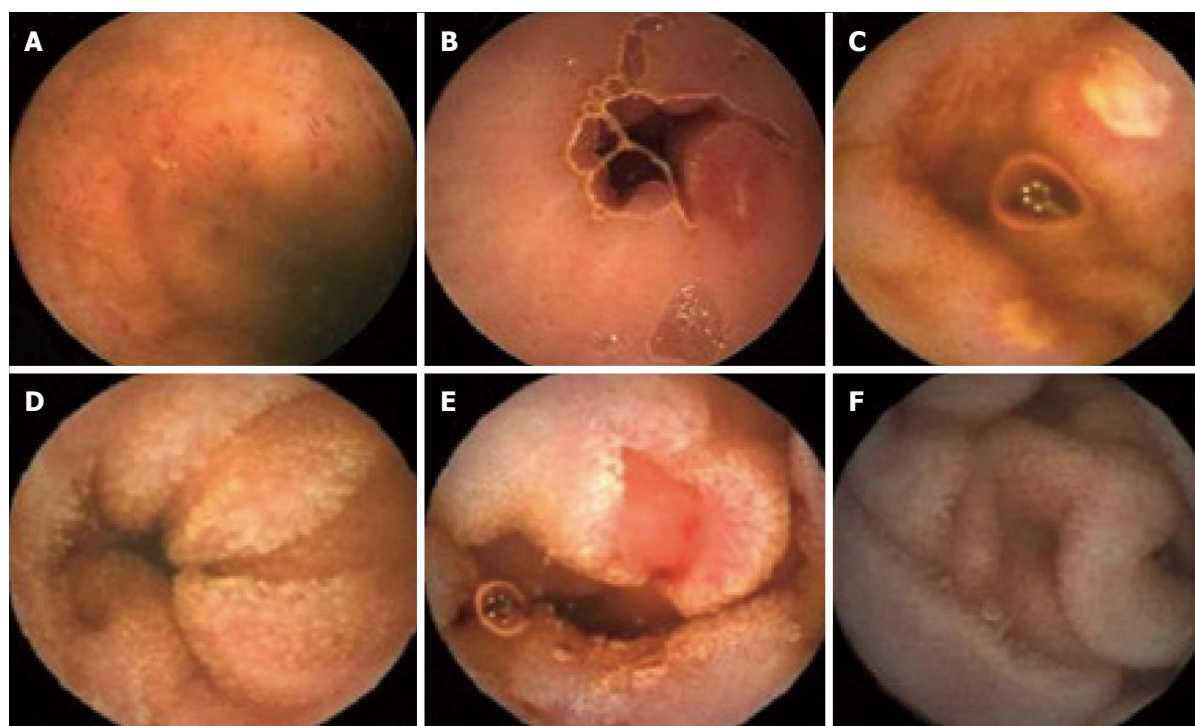
Figure 4 Representative examples of mucosal breaks observed during this study. A: Petechiae; B: Erosion; C: Ulcer; D: Lymphangiectasis; E: Denuded area; F: Normal.

Table 7 Number of small bowel mucosal breaks per tertile along the length of the small bowel				
	First	**Second**	**Third**	**P value[1]**
NSAID-muscovite group	0.8 ± 2.7	0.4 ± 1.3	1.3 ± 3.5	0.939
NSAID control group	2.8 ± 4.2	2.4 ± 4.1	5.9 ± 7.4	0.027
P value[2]	0.021	0.191	0.006	

[1]Calculated by using the Friedman χ^2 test; [2]Calculated by using the Wilcoxon signed rank test. NSAID: Nonsteroidal anti-inflammatory drug.

in Table 7. We observed no statistically significant difference in the distribution of small intestinal mucosal breaks across intestinal tertiles in the NSAID-muscovite group ($P = 0.939$). On the contrary, we observed a significant difference in the distribution of mucosal break across the tertiles of the small bowel in the NSAID control group ($P = 0.027$). Moreover, within each tertile, the difference between the NSAID-muscovite group and NSAID control group was statistically significant in the first and third tertiles, with the exception of the second tertile, probably due to fewer mucosal breaks observed in this tertile.

Complications encountered

Mild diarrhea during the first few treatment days was reported in 4 participants in the NSAID control group. We chose to keep these participants in the study, however, for the period of this study due to the mild nature of their symptoms. The remaining subjects experienced no complications for the duration of the study.

DISCUSSION

Subjects in the NSAID-muscovite group, who received muscovite in addition to diclofenac and omeprazole, had five-fold fewer number of small intestinal mucosal breaks after two weeks of treatment in comparison to the NSAID control group (2.5 *vs* 11.1, $P = 0.015$). In addition, participants in the NSAID-muscovite group were associated with a significantly lower percentage of subjects with one or more mucosal breaks (31.3% *vs* 71.4%, $P = 0.028$). Moreover, subjects in the NSAID-muscovite group had significantly lower injury severity of the small bowel in comparison to the NSAID control group. While 43% (6/14) of the NSAID control subjects had three or more ulcers, only 6% (1/16) of subjects in the NSAID-muscovite group had three or more ulcers ($P = 0.017$). In our study, we observed various NSAID-induced small bowel damages, such as petechia, erosions, ulcers, denuded areas or lymphangiectasis. Co-administration of muscovite resulted in a lower mean number of erosions and ulcerations induced by the short-term administration of NSAIDs. Although we did not observe a protective effect of muscovite against all observed intestinal damages, to the best of our knowledge, this study for the first time demonstrated by CE that treatment with muscovite could prevent or attenuate the severity of small bowel injury induced by some forms of NSAID. To determine if the treatment had a localized effect on any portion of the small bowel, we also conducted a post-hoc analysis of the distribution of mucosal breaks across the intestinal tertiles. There was a significant difference in the muco-

sal break distribution across the intestinal tertiles in the NSAID control group ($P = 0.027$).

It has been well established regarding the use of NSAIDs and the risk of small bowel damage and complications. Our results (71% of subjects in the NSAID control group developed NSAID-induced small-intestinal injuries; 57% developed ulcers) are consistent with findings recently reported for the small bowel from a video CE study in arthritis patients. Using CE, one study[3] showed that new intestinal damages developed in 68% of healthy subjects who received NSAIDs for 2 wk[2]. Another study[10] indicated that 55% of participants developed small bowel damages after the NSAID naproxen was administered for two weeks, with a mean of 2.99 mucosal breaks in each participant. In the majority of the end points measured in this study, the NSAID-muscovite group was statistically significantly different from the NSAID control group.

Although the cause of intestinal injury is not well understood, it is hypothesized that an aberrant increase in intestinal permeability promotes susceptibility to NSAID-induced inflammation and damage in the small intestine. As a type of traditional Chinese medicine, muscovite has served in the management of gastric diseases in China for many years. Pharmacological studies have confirmed that the layered structure of muscovite, with natural and special physical properties, may uniformly coat the surface of the gastric mucosa through the stimulation of mucus secretion to enhance intestinal mucosal barrier function. Alternatively, muscovite may effectively protect the mucosa by reducing the amount of direct contact with harmful luminal factors (*e.g.*, drugs, bile and various enzymes), thereby reducing membrane permeability. In addition, previous research has also shown that muscovite can effectively stimulate secretion of endogenous epidermal growth factor, which is known to promote mucosal repair and healing[15-17].

Many studies found that administration of omeprazole is ineffective in preventing injury in the small intestine[3,18]. In contrast, celecoxib, a cyclooxygenase-2 inhibitor, could effectively reduce the number of mucosal breaks each participant and the percentage of participants with one or more mucosal break[10]. Because cyclooxygenase-2 inhibitors may be associated with an increased risk of adverse cardiovascular events, many clinicians prefer to prescribe the traditional NSAIDs in combination with PPIs instead of cyclooxygenase-2 inhibitors in the management of NSAID-induced GI damages. Previously, no therapeutic agents existed to protect against NSAID-induced small bowel injury. Our paper broadens the understanding of the impacts of NSAIDs in the small bowel injury and explores the mechanisms of administration of traditional Chinese medicine (muscovite) on small bowel health. We found that participants who received muscovite treatment had a significantly lower number of small bowel mucosal break compared with those who received NSAIDs alone.

Although our study found that administration of muscovite could effectively prevent small intestinal damages induced by NSAIDs, some potential limitations should be mentioned. First, sample size of our study was relatively small and only healthy volunteers were included. Second, the short-term administration of NSAIDs and muscovite is not a typical course of treatment. In the clinical setting, patients often require long-term administration of NSAIDs. Third, our study had an inherent bias against neutrality because of its open-label trial design character. So, future trials with larger sample sizes are required to further evaluate the beneficial effect of muscovite identified in the present study.

COMMENTS

Background
Non-steroidal anti-inflammatory drugs (NSAIDs), one of the most commonly used classes of drugs worldwide, are widely accepted for their anti-inflammatory and analgesic properties. Although NSAIDs are beneficial in reducing pain and inflammation, they may induce fatal complication, such as ulcerations, perforation, bleeding or diaphragm-like strictures.

Research frontiers
Due to the increased use of aspirin and NSAIDs and the introduction of capsule endoscopy (CE), a new diagnostic modality, NSAID-induced enteropathy has gained much attention. NSAID-induced lower gastrointestinal (GI) injury is more common than NSAID-associated gastropathy, and has been underestimated or ignored in clinical practice prior to the wide use of CE.

Innovations and breakthroughs
This is the first try to use the traditional Chinese medicine (muscovite) to prevent NSAID-induced enteropathy in clinical trial. This study broadens the understanding of the impacts of NSAIDs in the small bowel injury and explores the mechanisms of administration of traditional Chinese medicine (muscovite) on small bowel health.

Applications
In this study, the authors found that participants who received muscovite treatment had a significantly lower number of small bowel mucosal break than participants who received NSAIDs alone, which means that traditional Chinese medicine (muscovite) co-therapy may have a brilliant future in preventing NSAID-induced lower GI injury.

Peer review
It is a very well designed and conducted study examining the potential protective effects of muscovite on the small bowel in those healthy volunteers taking NSAID for 2 wk.

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CASE REPORT

Significance of feeding dysfunction in eosinophilic esophagitis

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Abstract

Feeding dysfunction is a frequent presenting symptom of eosinophilic esophagitis (EoE). Here we present 3 children of various ages whose manifestations of EoE associated feeding dysfunction led to significant and life altering impact on their growth and development. Early identification of presenting symptoms of EoE will allow for prompt diagnosis and initiation of appropriate treatments. Recognition of salient features of dysfunction and treatment by feeding therapists and nutritionists led to symptom resolution and growth.

Key words: Eosinophilic esophagitis; Eosinophilic oesophagitis; Feeding dysfunction; Feeding therapy oral motor skills; Mealtime dynamics; Esophagitis; Oesophagitis

Core tip: Children with eosinophilic esophagitis may present with severe feeding dysfunction that manifests as growth disturbances. Feeding therapy can be an integral part of the treatment plan.

INTRODUCTION

Eosinophilic esophagitis (EoE) is a chronic esophageal disease characterized by reflux-like symptoms, dyspha-

gia or feeding dysfunction and eosinophil predominant esophageal inflammation[1-4]. It is estimated to occur in 4 of 10000 adults and children worldwide[2]. Here we present three children of different ages whose manifestations of EoE-associated feeding dysfunction led to life-altering impact on growth and development. Early recognition and treatment of EoE is necessary to prevent long-term complications of stricture and food impaction.

CASE REPORT

Case 1
A 20-mo-old boy presented for evaluation of nine months of chronic feeding refusal, being a "picky eater" and vomiting. Progressive reduction in solid food intake led to slow weight gain. Physical examination revealed mild wasting (83% ideal weight for height). Clinicopathological evaluation confirmed the diagnosis of EoE and treatment was initiated (Table 1). Feeding evaluation identified refusal to eat meats, vegetables or fruits unless pureed and preference for liquids. Food allergies to egg and peanut were identified. Parental frustration centered on the inability to introduce new foods, low volume of intake and lengthy mealtimes. After medical and feeding therapy, he gained weight (95% ideal weight for height) and vomiting resolved. Family feeding therapy improved the patient's oral motor skills allowing him to increase food texture variety and caloric intake, develop appropriate mealtime behaviors and add new foods. He participated in mealtimes with positive behaviors thus reducing caregiver frustration.

Case 2
A 4-year-old boy presented with 2 years of intermittent food refusal, vomiting and gagging associated with eating. Treatment with lansoprazole reduced his vomiting but did not resolve other symptoms. He had a history of asthma. Physical examination and growth were normal (110% ideal weight for height). A clinicopathological diagnosis of EoE was made and medical treatment started (Table 1). Feeding evaluation revealed solid food refusal, preference for soft foods and significant mealtime anxiety that resulted in > 1 h-long meal times. Clinical evaluation revealed problems chewing highly textured foods (meats, breads). Eating behaviors and symptoms lead to stressful family dynamics and mealtimes. Individual feeding therapy sessions integrated new foods into his diet, reduced food refusal behaviors, decreased mealtime length, diet expansion and skill acquisition fostering positive mealtimes.

Case 3
A 15-year-old girl presented with a 9-year history of solid food dysphagia. She avoided meat, ate slowly, and limited her diet to foods that did not "get stuck". Physical examination was notable for wasting (80% ideal weight for height). A clinicopathological diagnosis of EoE was made and treatment initiated. She had a history of cat allergies and allergic rhinitis (Table 1). Feeding evaluation revealed

that she used liquids to "wash" food down, avoided meat and breads, took small bites, preferred foods with soft textures and experienced prolonged mealtimes. To avoid embarrassment, she told friends she was a vegetarian and limited social engagements. Food allergies to sesame, nuts and bananas were identified. Nutritional intervention focused on achieving appropriate weight gain. Treatment with swallowed topical steroid (fluticasone) and food restrictions of sesame, nuts and bananas were started, leading to resolution of symptoms and esophageal eosinophila after 2 mo later. Despite resolution of dysphagia and esophageal eosinophilia after two months of treatment, feeding behaviors and anxiety persisted. Feeding therapy was initiated to achieve appropriate chewing and swallowing skills and develop strategies for trying new foods in social settings. She incorporated 15 to 20 new foods into her diet. Weight improved (90% ideal weight for height). Her anxiety with social eating resolved and she was able to eat all foods, including meats.

DISCUSSION
Since children develop feeding skills during infancy and throughout childhood, any disruption of this pattern, caused by discomfort or inflammation, can result in life changing, maladaptive eating behaviors. These feeding disturbances can occur at different ages and stages of childhood development (Table 2). In this regard, a limited number of reports have identified the spectrum of feeding dysfunction associated with EoE. Cross-sectional studies determined that feeding dysfunction occurs in 14% to 58.9% of children with EoE[3,5]. Pentiuk *et al*[6] describe a number of infants and toddlers presenting to their feeding specialty clinic who were ultimately diagnosed with EoE. However the importance of early recognition and feeding therapy in the overall successful evaluation and treatment of patients with EoE has not been thoroughly emphasized. These cases provide examples of the critical importance of the recognition of feeding dysfunction as a cardinal symptom of EoE as well as the potential need for, and impact of, feeding therapy necessary for some children with EoE.

The first patient demonstrates classic feeding problems observed in infants and toddlers with chronic esophagitis. Food refusal behaviors delay acquisition of age appropriate feeding skills. These children often present as "drinkers and food refusers." Feeding therapy encouraged development of oral motor skills and reduction in maladaptive learned feeding behaviors. Feeding therapy, concurrent with effective medical therapy, led to improvement in feeding behaviors, accelerated weight gain and reduced family mealtime stress.

The second case demonstrates how chronic pain led to feeding dysfunction and development of maladaptive coping in a pre-school child. In this scenario, development of mature eating skills was stunted and family mealtime dynamics disrupted. Feeding therapy facilitated increased oral intake and normalized mealtime dynamics,

Table 1 Summary of clinical data

	Age	Symptom duration at presentation	Presenting symptoms	Diagnostic endoscopy[1]	Treatment	Histologic resolution	Feeding therapy
1	20 mo	9 mo	Vomiting, feeding refusal	Edema and exudate; up to 70 eos/hpf	OVB 0.5 mg BID; ADED	Normal; no eos/hpf	Weekly individual sessions
2	4 yr	2 yr	Vomiting, abdominal pain, feeding refusal	Furrows and exudate; up to 60 eos/hpf	Fluticasone 44 ug 2 puffs BID swallowed	Normal; no eos/hpf	Individual sessions followed by group sessions
3	15 yr	9 yr	Solid food dysphagia	Ringed esophagus; up to 46 eos/hpf	Fluticasone 220 ug 2 puffs BID swallowed, ADED	Furrows; up to 4 eos/hpf	Bimonthly individual sessions

[1]Gross and histologic appearance. All diagnostic endoscopies were performed after 8 wk of age appropriate high dose proton pump inhibitor treatment to effectively exclude gastroesophageal acid reflux disease. eos/hpf: Eosinophils per high power field; OVB: Oral viscous budesonide; ADED: Allergen directed elimination diet, as identified by immunocap and skin prick testing.

Table 2 Common feeding dysfunction and clinical gastrointestinal presentation seen in eosinophilic esophagitis by age

	Infant/toddler	School age	Older child/adolescent
Feeding presentation	Liquid and food refusal, delayed oral feeding skills, low volume of intake, grazing behaviors	Food refusal, poor acceptance of new foods, preference for liquid and soft diet, low variety in diet, slow pace of eating, need for prodding to eat	Preference for liquid and soft diet, low variety in diet, fear and anxiety at mealtimes
Gastrointestinal presentation	Vomiting, irritability, pain	Abdominal pain, vomiting	Dysphagia, heartburn

even before histologic normalization.

The third case revealed how EoE contributed to maladaptive feeding behaviors, malnutrition and social disruption in a teenager. Dysphagia led to fear and anxiety about eating and social isolation. Maladaptive behaviors led to reduced intake and malnutrition. Feeding therapy was required to reduce anxiety and improve eating, even after histologic normalization and clinical improvement.

After medical and feeding treatments, each patient either developed previously absent skills or recovered skills that facilitated growth. Major goals of EoE treatment are reduction in esophageal inflammation and optimization of growth and development. Our report emphasizes that, in some children with EoE, early identification and treatment of feeding dysfunction with feeding therapy is key to meeting these goals as evidenced by their improvement in feeding behaviors, intake and growth. Gastroenterologists may miss initial historical features of feeding dysfunction and not recognize the full impact of therapeutic interventions. Individualized or group feeding therapy that includes parents and other caregivers provides necessary immediate tools and long-term feeding strategies.

EoE is a chronic disease that can present with feeding dysfunction. Early recognition of feeding problems as a diagnostic clue for EoE is important to potentially prevent esophageal remodeling and functional sequelae such as dysphagia and food impactions[7,8]. Institution of age appropriate medical and feeding treatments is critical for children of all ages.

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COMMENTS

Case characteristics
Three children with eosinophilic esophagitis presenting with severe feeding dysfunction.

Differential diagnosis
Exclusion of Gastroesophageal reflux disease with treatment with proton pump inhibition.

Pathological diagnosis
Esophageal eosinophilia with greater than 15 eosinophils/high power field and exclusion of other causes of inflammation.

Treatment
Topical corticosteroids, diet restriction and feeding therapy were used to induce symptomatic and histological remission.

Related reports
Feeding dysfunction as an initial manifestation of eosinophilic esophagitis and feeding therapy as an important part of a treatment plan are under recognized.

Experiences and lessons
This case series is the first to document severe feeding dysfunction in children with eosinophilic esophagitis of various ages who received benefit from feeding therapy.

Peer review
This article will increase awareness of feeding dysfunction as a manifestation of eosinophilic esophagitis and the positive impact of feeding therapy.

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INSTRUCTIONS TO AUTHORS

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access (OA) journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1353 experts in gastroenterology and hepatology from 68 countries.

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

Organization as author

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Both personal authors and an organization as author

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

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

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

Issue with no volume

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

Books

Personal author(s)

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

Chapter in a book (list all authors)

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

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

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

Conference paper

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

Electronic journal (list all authors)

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

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

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