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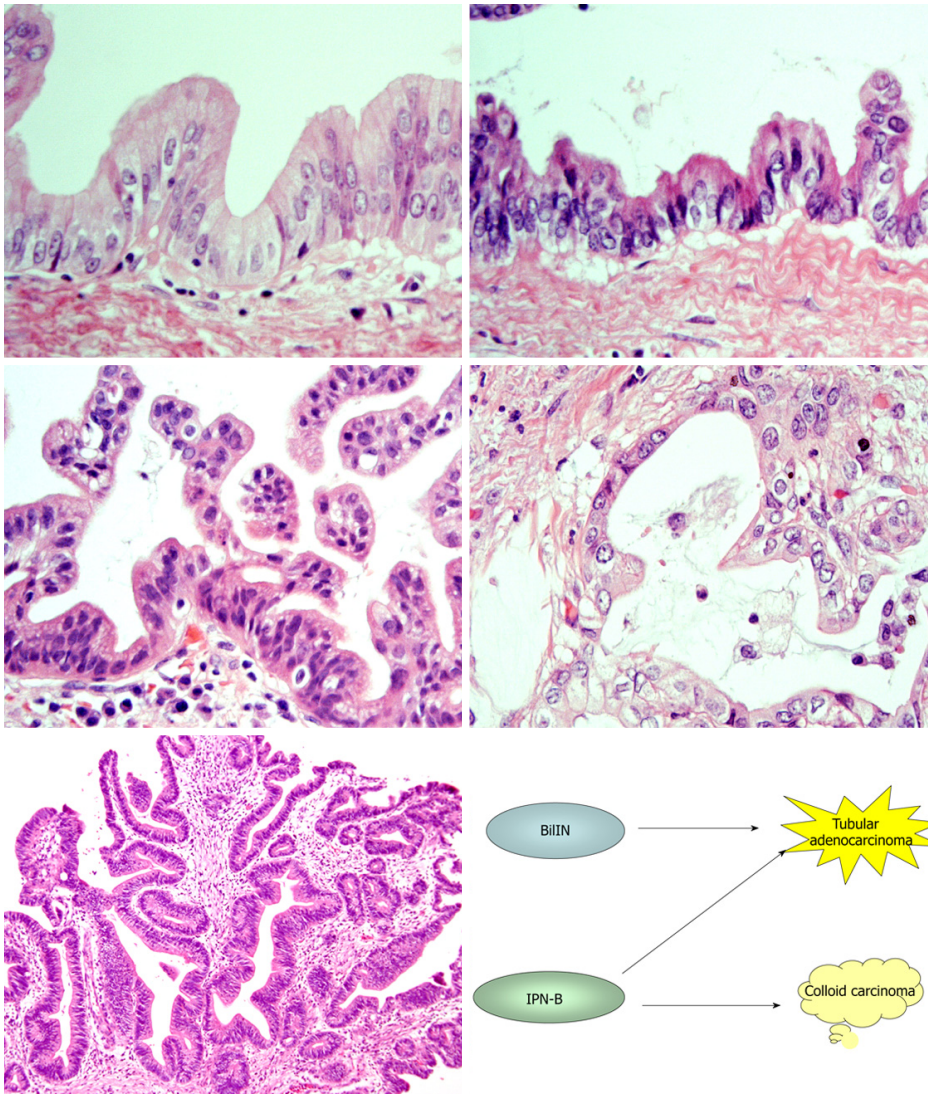

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EDITORIAL

What is the purpose of launching *World Journal of Hepatology*?

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Abstract

The first issue of *World Journal of Hepatology* (*WJH*), whose preparatory work was initiated on September 23, 2008, will be published on October 31, 2009. The *WJH* Editorial Board has now been established and consists of 213 distinguished experts from 29 countries. Our purpose of launching *WJH* is to publish peer-reviewed, high-quality articles *via* an open-access online publishing model, thereby acting as a platform for communication between peers and the wider public, and maximizing the benefits to editorial board members, authors and readers.

Key words: Maximization of personal benefits; Editorial board members; Authors; Readers; Employees; *World Journal of Hepatology*

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INTRODUCTION

I am very pleased to announce that the first issue of *World Journal of Hepatology* (*World J Hepatol*, *WJH*, ISSN 1948-5182, DOI: 10.4254) will be published on October 31, 2009. Originally, the journal was titled *Liver Cancer Review Letters* when preparatory work was initiated on September 23, 2008, and retitled *Liver Disease Review Letters* at a later date. The *WJH* Editorial Board has now been established and consists of 213 distinguished experts from 29 countries.

The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. To realize these desired attributes of a journal and create a well-recognized journal, the following four types of personal benefits should be maximized.

MAXIMIZATION OF PERSONAL BENEFITS

The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others.

Maximization of the benefits of editorial board members

The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution.

Maximization of the benefits of authors

Since *WJH* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJH* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading.

Maximization of the benefits of readers

Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion[1].

Maximization of the benefits of employees

It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal[2,3]. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality articles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

CONTENTS OF PEER REVIEW

In order to guarantee the quality of articles published in the journal, *WJH* usually invites three experts to comment on the submitted papers. The contents of peer review include: (1) whether the contents of the manuscript are of great importance and novelty; (2) whether the experiment is complete and described clearly; (3) whether the discussion and conclusion are justified; (4) whether the citations of references are necessary and reasonable; and (5) whether the presentation and use of tables and figures are correct and complete.

SCOPE

The major task of *WJH* is to report rapidly the most recent results in basic and clinical research on hepatology, including: liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology.

COLUMNS

The columns in *WJH* will include: (1) Editorial: to introduce and comment on major advances in rapidly developing areas and their importance; (2) Frontier: to review recent developments, comment on current research status in important fields, and propose directions for future research; (3) Topic Highlight: this column consists of three formats, including: (a) 10 invited review articles on a hot topic; (b) a commentary on common issues associated with this hot topic; and (c) a commentary on the 10 individual articles; (4) Observation: to update the development of old and new questions, highlight unsolved problems, and provide strategies for their resolution; (5) Guidelines for Basic Research: to provide Guidelines for basic research; (6) Guidelines for Clinical Practice: to provide guidelines for clinical diagnosis and treatment; (7) Review: to review systemically the most representative progress and unsolved problems, comment on current research status, and make suggestions for future work; (8) Original Article: to report original and innovative findings; (9) Brief Articles: to report briefly on novel and innovative findings; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: to discuss and reply to contributions published in *WJH*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: to introduce and comment on quality monographs; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities on basic research and clinical practice.

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EDITORIAL

Recent insights on risk factors of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is a disease prevalent in many populations worldwide. It initiates many economic and health problems in management modalities and leads to increasing mortality rates. Worldwide, trials have attempted to discover specific early markers for detection and prediction of the disease, hoping to set a more precise strategy for liver cancer prevention. Unfortunately, many economic, cultural and disciplinary levels contribute to confounding preventive strategies. Many risk factors contribute to predisposition to HCC, which can present individually or simultaneously. Previous articles discussed many risk factors for hepatocellular carcinogenesis; however, most of them didn't consider collectively the most recent data relating to causes. In this article, the pathogenesis and risk factors of HCC are discussed. Most of the intermediary steps of HCC involve molecular and transcriptional events leading to hepatocyte malignant transformation. These steps are mainly triggered by hepatitis B, C or transfusion-transmitted virus, either alone, or with other factors. Diabetes seems to be a major contributing risk factor. Schistosomiasis, a blood infestation, mostly affects Nile basin inhabitants leading to bladder, renal and hepatic cancers. Alcoholism, food and water pollutants and some drugs can also lead to HCC. Additionally, some hereditary diseases, as hemochromatosis, α-1-antitrypsin deficiency and tyrosinaemia are known to lead to the development of HCC, if not well managed.

Key words: Hepatocellular carcinoma; Hepatitis C virus; Hepatitis B virus; Transfusion-transmitted virus; Schistosomiasis; Risk, Alcoholism and hereditary diseases

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INTRODUCTION

Hepatocellular carcinoma (HCC) is ranked to be the most common cancer in many countries[1]. Recently, HCC was reported to be the fifth most common cancer in males, the eighth common cancer in females and about 560 000 cases are discovered per year, more than 80% of which occur in the developing countries. Having very poor prognosis, it represents the third leading cause of cancer death worldwide; more than one-half of them in China. Generally, HCC is more frequent in men than in women and the incidence increases with age[2]. Like other cancers, it is a multistep process, involving many genetic alterations, which eventually lead to malignant transformation of hepatocytes. Most liver diseases lead to cirrhosis. Within 15-40 years, chronic hepatitis leads to cirrhosis. Mostly, HCC develops among 70%-90% of cirrhotic patients, while only 10% of HCC patients have a non-cirrhotic liver, or even have inflammatory lesions[2]. According to the WHO mortality database of the early 1980s, the highest rates were found in Mexico and Chile, France, Italy, Portugal, Austria,

Hungary and Romania. Unfortunately, figures are rising in many European countries, including, in the UK, Wales and Scotland, possibly due to increased consumption of alcohol[3]. Alcoholic liver diseases and hepatitis C infection, being primary etiologies for liver cirrhosis, are the major causes of the rising HCC mortality rates[4].

PATHOGENESIS OF HUMAN HCC

Being implicated in more than 70% of HCC cases world wide, liver cirrhosis is the major risk factor for HCC development. Liver carcinogenesis may last for decades, through progressive accumulation of different genetic alterations eventually leading to malignant transformation. Thus, chronic liver injury initiates increased liver cell turnover, triggering oxidative DNA damage and inflammatory events. This leads to formation of dysplastic and macroregenerative nodules, which are considered to be neoplastic[5].

A-MOLECULAR PATHWAYS AND THEIR POSSIBLE RELATIONSHIP TO HCC

The underlying steps in human hepatocarcinogenesis: there are at least four molecular pathways that regulate either proliferation or death.

1-Irregular expression of β-catenin

β-catenin is a nuclear protein that regulates the cell cycle. Its irregular expression, resulting from β-catenin gene mutations, is implicated in HCC. In addition, alteration to the Wnt signaling pathway plays a role in more than 50% of HCCs[6]. Wnt molecules are a large family of cysteine-rich secreted glycoproteins that control development in organisms, ranging from nematodes to mammals. Interestingly, accumulated intra-nuclear β-catenin form complexes with proteins such as Wnt ligands and Frizzled receptors, leading to unrestricted cell cycling[7].

2-Up-regulation of many growth factors

Insulin-like growth factor (IGF), insulin receptor substrate 1, hepatocyte growth factor (HGF) and transforming growth factor β (TGF-β) have been implicated in the development of HCC[8].

3-Transformation from pre-neoplastic to HCC nodules

HCC is a highly vascular tumor, always accompanied by neo-vascularization. Thus, overexpression of angiogenic factors, vascular endothelial growth factor (VEGF) and angiopoietin-2, is another pathway for HCC genesis[9,10].

4-Mutations in transcription factors controlling the cell cycle

Transcription factors such as phospho-retinoblastoma (pRb), P53, TGF-β and β-catenin participate in hepatocellular carcinogenesis[11]. Mutations in these factors deprive the cell control over the cell cycle, leading to uncontrolled mitosis and cancer.

B-RISK FACTORS THAT LEAD TO HCC

HBV infection

This DNA virus is the most frequent etiology of liver cancer. There is strong epidemiological evidence correlating HCC to HBV infection. This was shown by positive results in HCC patients for both HB surface antigen (HBs Ag) and HB core antibodies (HBc antibodies) or both together[12]. However, patients with negative hepatitis B serum markers, although showing symptoms of chronic hepatitis or cirrhosis, were proved to have active intrahepatic replicating virus. This is conventionally known as occult HBV infection[13].

HCV infection

In developing countries, the major concern in HCC is chronic HCV infection. Chronic HCV infection mostly leads to hepatic cirrhosis before developing HCC[14]. Additionally, occult HCV was also reported in patients with chronic un-explained hepatitis[15]. Thus, both occult HBV and HCV infections contribute to HCC prevalence. These can be detected by the invasive biopsy technique, which is the sole diagnostic tool in occult uncertain infections.

Generally, the prevalence of HCV-infection is accepted to be a major morbidity factor in hepatic carcinogenesis. In developing countries, the mode of transmission of HCV is diverse. Old habits of injection, shaving, circumcision, blood transfusion, labor and surgical viral transmission, frequently created many infected generations who carry the infection for many years, although the modes of transmission were greatly minimized by hygienic and cultural development. However, these old-infected populations constitute classic candidates for long standing infection, cirrhosis and HCC. HCV is a member of the Flaviviridae family of enveloped, positive-stranded RNA viruses, genus Hepacivirus. It is a completely cytoplasmic-replicating virus that induces oncogenic transformation[16]. An increasing body of evidence suggests that HCV has a direct pathway in promoting malignant hepatocyte transformation. However, it also now established that many viral proteins are implicated in malignant transformation and HCC development. Of these proteins, core proteins, NS3, NS4, were shown to have transformation potential in tissue culture[17-20]. These viral proteins, in addition to the viral RNA, interact with many host-cell factors, while still regulating the viral life cycle. They modulate host-cell activities such as cell signaling, transcription, transformation, apoptosis, membrane rearrangement, vesicular trafficking and protein translation. This ultimately misleads the host transcription factors, disturbing cell mitosis and protein synthesis, leading to carcinogenesis[2]. On the other hand, the HCV core has immunosuppressive activities through interaction with the complement receptor C1qR on the T cells leading to chronic infection[21].

Transfusion-transmitted virus

Another possible risk factor for HCC, found in patients

with HCV-related liver disorder is transfusion-transmitted virus. These viral DNA traces were only discovered by fine *in situ* PCR in liver biopsies, which could be described to be neither HBV nor HCV material[22,23].

Diabetes mellitus

Liver cirrhosis, which is a functional liver damage (characterized by a decrease in serum albumin level below 4 g/dL and increased prothrombin time), is always higher in HCC patients with diabetes, than among those without a history of diabetes. Thus, there is a positive correlation between the history of diabetes mellitus and HCC, which was not confounded by any other HCC risk factor, as observed by Lagiou and co-workers[24]. A number of possible mechanisms explained this association. Most non-insulin dependent diabetics show hyper-insulinemia. Thus, insulin or its precursors may interact with liver cells to stimulate mitogenesis or carcinogenesis[25,26]. Another possible pathway is that a p53 mutation (an apoptotic factor) was noticed frequently in HCC patients with diabetes rather than non-diabetics, this could provide an evidence for a molecular mechanism involving this common association[27].

Hereditary hemochromatosis

Hereditary hemochromatosis is an autosomal recessive condition characterized by excessive iron deposition in hepatocytes due to an increased intestinal absorption. Thus, liver disease is the commonest cause of death in patients with hereditary hemochromatosis[28]. Among hemochromatotic patients, 6% of men and 1.5% of women are at absolute risk of liver cancer[29]. However, a cross-sectional study showed that progression to HCC among hemochromatotic patients is mostly variable from one population to another, depending mainly on exposure to environmental factors that synergize the current underlying gene mutation[30].

Schistosomiasis among Egyptian Nile basin population

Many cross-sectional studies on wide Egyptian sectors frequently correlated HCV infection and intravenous treatment for schistosomiasis, which is a common parasitic infestation frequently constituting a serious predisposing factor for hepatic fibrosis[31]. Many HCC cases were also diagnosed among long standing bilharziasis.

Exposure to chemical carcinogens

Environmental pollutants such as aflatoxin B, a product of mold commonly contaminating badly stored foods, as well as insecticides, were reported to be classical sources for hepatocarcinogenesis[32]. Other known chemical carcinogens are chlorination byproducts in drinking water. Uncontrolled water chlorination converts many organic traces in water into dangerous intermediates, such as di-and tri- chloroacetic acids, which are experimentally known to induce HCC[33]. Additionally, a rarely encountered chemical contaminant to drinking water, the algal toxin, microcystin, which is found in pond-ditch waters, can induce primary liver cancer[34]. However, many other chemical contaminants, such as solvents, food additives, drugs and hormones, are thought to contribute to HCC. Recent studies strongly suggested that bile acids might be pro-inflammatory and oncogenic agents. Thus, chronic exposure to bile acids plays an important part in inflammation and hepato- and cholangiocellular carcinogenesis[35].

Alcoholism

Alcohol is a very common source for steatohepatitis (fatty liver), cirrhosis and eventually HCC[36]. In developed countries, alcohol drinking seems to be the most common source for HCC. Alcohol either directly initiates HCC after its oxidation into acetaldehyde, which is genotoxic, or indirectly through the development of cirrhosis[37]. Epidemiological studies suggested a strong synergistic effect of alcohol on both HBV and HCV infections in developing HCC[38].

Congenital disorders

Alpha-1-antitrypsin deficiency and tyrosinemia might be complicated by the development of HCC[39]. Thus, dietary or pharmacological management of hereditary tyrosinemia might offer a strategy for prevention of HCC in these cases[40]. On the other hand, alpha-1-antitrypsin is an acute-phase protein that is produced by liver cells. Hereditary deficiency of this protein is mostly due to liver production of the abnormal protein that cannot be released into the plasma. Accumulation of the protein in hepatocytes can lead to liver damage. This can trigger hepatitis in neonates, end-stage liver disease, cirrhosis and HCC in adults[41].

Recent insights on laboratory detection of HCC

Recently, I published a review of the laboratory markers useful in diagnosing HCC, either specific RNAs or serum proteins. These included molecular markers such as hepatoma specific alpha fetoprotein (HS-AFP) mRNA, hepatoma specific gamma glutamyl transferase (HS-GGT) mRNA, transforming growth factor β1 (TGF-β1) mRNA, insulin-like growth factor-Ⅱ (IGF-Ⅱ) mRNA, heat shock protein (HSP) and methylated apoptotic factors (such as p53-mRNA). Serum markers such as AFP, alpha-L-fucosidase (AFU), GGT, TGF-β1, IGF-Ⅱ, anti-p53 antibodies and des-gamma-carboxy prothrombin (DCP) in addition to less common markers such as r-glutamyl transpeptidase (r-GT), tumor necrosis factor alpha (TNF-α), pancreatitis-associated protein (PAP), serine-threonine kinase 15 (STK-15) and plasma glutamate carboxypeptidase (PGCP) were also studied.

The most important conclusion was that the use of AFP, AFU and methylated p53-mRNA together could give a 100% early prediction of HCC development in risky subjects. This short panel of three markers is the recommended to ensure optimal HCC prediction with the highest priority to other studied markers[42].

CONCLUSION

Initiation of HCC is often mediated by complex mole-

cular and transcriptional cascades leading to hepatocyte malignant transformation. These molecular changes include, irregular expression of β-catenin, up-regulation of many growth factors, transformation from pre-neoplastic to HCC nodules and mutations in transcription factors controlling the cell cycle. This cascade is mostly triggered by individual or confounding risk factors. These risk factors include hepatitis B or C, blood transfusion transmitted viruses, diabetes, Schistosomiasis, alcohol intake/alcoholism, food and water pollutants, as well as exogenous and endogenous carcinogenic chemicals. Some hereditary diseases such as hemochromatosis, α-1-antitrypsin deficiency and tyrosinaemia might lead to the development of HCC if not properly managed.

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RESEARCH FRONTIER

Future perspectives on the treatment of hepatocellular carcinoma with cisplatin

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Abstract

Hepatocellular carcinoma (HCC) is the commonest primary liver malignancy. Its incidence is increasing worldwide. Surgery, including transplantation resection, is currently the most effective treatment for HCC. However, recurrence rates are high and long-term survival is poor. Conventional cytotoxic chemotherapy has not provided clinical benefit or prolonged survival for patients with advanced HCC. Cisplatin (CDDP) is a key drug for the standard regimens of various cancers in the respiratory, digestive and genitourinary organs. Recently, several encouraging results have been shown in using CDDP in the treatment of advanced HCC patients. This review examines current knowledge regarding the chemotherapeutic potential of CDDP.

Key words: Hepatocellular carcinoma; Hepatic arterial infusion; Cisplatin

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INTRODUCTION

It has become possible to identify a group of patients with chronic liver disease who are at a high risk of developing hepatocellular carcinoma (HCC)[1]. In addition, advances in diagnostic imaging have allowed relatively early diagnosis of HCC. However, it is still not rare to find patients in whom HCC is diagnosed at an advanced stage of the disease. For the treatment of hepatocellular carcinoma, various treatments, including hepatectomy, transcatheter hepatic arterial embolization (TAE), transcatheter hepatic arterial chemoembolization (TACE), percutaneous ethanol injection therapy (PEIT), percutaneous microwave coagulation therapy (PMCT) and percutaneous radiofrequency ablation (PRFA), were performed either singly or in combination. Thus, local control has been attempted taking into account the localization of the tumor, location of the lesion, and the hepatic reserve. On the other hand, it is necessary to apply effective chemotherapy to patients who develop recurrence after these treatments for local control of the disease, as also those with highly advanced disease. In most previous studies, either only a small number of patients were included or there was no control group, resulting in the difficulty of reaching a consensus in the establishment of a standard treatment. A group, led by Dr Makuuchi, was formed with the purpose of issuing guidelines for the diagnosis and treatment of liver cancer by "Development of Evidence-based Guideline Diagnosis and Treatment" of Ministry of Health, Labor and Welfare. "Evidence-based Guidelines for the Diagnosis and Treatment of Hepatocellular Carcinoma, version 2005"[2] were published in March 2005. These are the first established guidelines for the diagnosis and treatment of HCC based on the principle of evidence-based medicine. According to the guidelines, currently, and based on scientific evidence, there are no chemotherapies that can be recommended. HCC is resistant and relatively insensitive to chemotherapy, rarely showing response to treatment. Therefore, no standard regimens have been established yet.

This study is focused on cisplatin, which is now attracting much attention among anticancer drugs, used for solid cancers, as a powerful agent that can be used for the treatment of HCC by arterial infusion. Future perspectives in relation to the use of this agent are outlined, based on a review of all the studies reported to date in the literature.

OVERVIEW OF CISPLATIN

In 1965, a bacteriologist, Barnett Rosenberg, found that a platinum compound, eluted from platinum electrodes used in his experiments, had an inhibitory effect on the growth of *Escherichia coli*. Later, various platinum compounds were examined for their antitumor activity, hoping to find a drug that would inhibit the division of cancer cells, characterized by rapid proliferation. Subsequently, cisplatin (cis-diamminedichloroplatinum; CDDP) was identified as a compound with high antitumor activity.

In 1972, clinical research on CDDP was started at the US National Cancer Institute (NCI), and the usefulness of CDDP as an antineoplastic agent was first confirmed in the treatment of malignant tumors of the urinary system. Currently, CDDP is a key drug in standard regimens for the treatment of various cancers, including of the respiratory, digestive and genitourinary systems[3].

CDDP exerts its action through the following mechanism. After entering the target cells, CDDP binds to the cellular DNA to form a covalent complex. The drug causes reversible alkylation of guanine and adenine, and forms intra- and interstrand cross-links in the DNA, thereby inhibiting elongation of DNA by DNA polymerase (inhibition of DNA transcription and replication). In addition, the formation of intrastrand cross-links results in changes in the conformation of the cells. These changes induce apoptosis and necrosis of the cancer cells, and underlie the antitumor effect of the drug. The anticancer effect of CDDP is characterized by both concentration-dependent and time-dependent features.

CDDP is mainly excreted *via* the kidney. The percentage of cumulative 24-h urinary excretion relative to the dose administered has been reported to be 30%-40%. Pharmacokinetic studies have revealed that the CDDP in the plasma rapidly binds to plasma proteins practically irreversibly, to become inactivated. Free CDDP (unbound to proteins) has been found at a minimally detectable levels 2 h after the end of administration, to fall below the detection limit 2-4 h later. Free CDDP exerts antitumor activity and, at the same time, accumulates in the proximal renal tubules to cause tubular impairment. Therefore, it is important to take measures against potential acute renal damage occurring in the presence of free CDDP in the blood within 2 h of the end of administration. Prophylactic measures against renal damage include hydration and forced diuresis to decrease the urinary CDDP concentration, aimed at minimizing the period of contact between CDDP and the renal tubules. Furosemide and/or mannitol are the commonly used diuretics to induce forced diuresis. Premedication with an antiemetic, such as a $5HT_3$ receptor inhibitor, is necessary, because CDDP has a strong emetic action.

CDDP is also known as a modulator of 5-fluorouracil (5-FU). It is well-known that 5-FU forms a covalent ternary complex with thymidylate synthase (TS). FdUMP is converted into the active form inside the cell in the presence of tetrahydrofolate, and inhibits the catalytic function of TS, thereby inhibiting DNA synthesis, which underlies the antitumor effect of 5-FU. CDDP acts on the cell membrane and inhibits the entry of methionine into the cells, leading to a reduction of the intracellular methionine pool. It has been postulated that this results in homocysteine methylation for the synthesis of methionine inside the cells. In relation to this synthetic reaction, the tetrahydrofolate pool increases to promote the formation of the covalent ternary complex and enhances DNA synthesis inhibition by 5-FU. CDDP exerts this effect, regardless of whether it is bound or unbound to plasma proteins.

SYSTEMIC CHEMOTHERAPY

Advanced HCC patients who are candidates to chemotherapy are those who are unlikely to respond to hepatectomy, RFA and/or TACE, have well-maintained liver function (Child-Pugh A, B) and a stable general condition (PS 0-2). Such patients would include those with (1) severe vascular invasion, (2) multiple intrahepatic lesions, and (3) distant metastases. No chemotherapeutic agent has been shown to exert consistently satisfactory antitumor effect against HCC, and most potentially effective drugs have been examined in pilot studies conducted on only limited numbers of patients. Among such drugs, the response rate to CDDP, given as systemic monotherapy, has been reported to be 15%[4], and multidrug regimens containing this agent may be expected to yield higher response rates. Arterial infusion chemotherapy, which allows higher concentrations of the drug to be achieved inside the tumor and thereby higher antitumor effect, has been reported to yield higher antitumor efficacy than systemic administration, and the reported response rates to arterial infusion regimens containing CDDP range from 41%-61%[5,6]. High efficacy of systemic chemotherapy with CDDP for HCC has not been reported, similarly to various other anticancer drugs. According to the available results, the response rate is 9.3%-17% for the intravenous administration of CDDP alone, and 10%-20% for the combined therapy. Thus, no satisfactory survival effect has been shown for any of the various regimens[4,7-17] (Table 1). However, Ikeda *et al*[12,13], who used three drugs (5-FU, mitoxantrone, CDDP), achieved relatively favorable results: a response rate of 27% (14/51), median survival time (MST) of 11.6 mo, and median progression-free survival of 4.0 mo. This seems to be a promising treatment regimen for patients of HCC with extrahepatic metastases.

Table 1 Systemic chemotherapy

Author	Country and region	Publication year	Treatment schedule	*n*	RR (%)	PFS (M)	MST (M)	1yrs (%)
Falkson *et al*[7]	USA	1987	CDDP 75 mg/sq q3w	35	< 17	-	3.2	-
Okada *et al*[4]	Japan	1993	CDDP 80 mg/sq q4w	26	15.4	-	-	-
Nagahama *et al*[8]	Japan	1997	CDDP	43	9.3	-	-	-
Ji *et al*[9]	Korea	1996	CDDP 60 mg/sq q4w IFN-α 3MU im for 3 mo	30	13.3	-	7.6	23.5
Leung *et al*[10]	China	2002	CDDP 20 mg/sq, d1-4 DXR 40 mg/sq, d1 5-FU 400 mg/sq, d1-4 IFN-α 5MU SC d1-4 q3w	149	16.8	-	7.1	-
Yang *et al*[11]	Taiwan	2004	CDDP 80 mg/sq d1 Mitoxantrone 6mg/sq d1 5-FU 450 mg/sq d1-5 q4w	63	23.8	2.5	4.9	-
Ikeda *et al*[12,13]	Japan	2008 (2005)	CDDP 80 mg/sq d1 Mitoxantrone 6 mg/sq d1 5-FU 450 mg/sq d1-5 q4w	82	22.0	3.2	11.2	43.5
Parikh *et al*[14]	India	2005	CDDP 70 mg/sq d1 GEM 1250 mg/sq d1, 8 q3w	30	20.0	4.1	4.8	27.0
Yeo *et al*[15]	China	2005	CDDP 20 mg/sq, d1-4 DXR 40 mg/sq, d1 5-FU 400 mg/sq, d1-4 IFN-α 5MU. SC d1-4 q3w	94	20.9	-	8.7	39.0
Kim *et al*[16]	Korea	2006	CDDP 60 mg/sq d1 EPI 50 mg/sq d1 UFT 400-600 mg/d PO 3w leucovorin 75 mg/d PO 3w q4w	53	16.9	2.7	5.7	-
Park *et al*[17]	Korea	2006	CDDP 60 mg/sq, d1 DXR 60 mg/sq, d1 Capecitabine 2 g/sq per day 2w q3w	29	24.1	3.7	7.7	-

PO: Per os; SC: Subcutaneous injection; im: Intramuscular injection; CDDP: Cisplatin; DXR: Doxorubicin; EPI: Epirubicin; 5-FU: 5-fluorouracil; UFT: Tegaful-uracil; GEM: Gemcitabine; IFN: Interferon; RR: Response rate; PFS: Progression free survival; MST: Median survival time; 1yrs: 1 year survival rate.

HEPATIC ARTERIAL INFUSION CHEMOTHERAPY WITH CISPLATIN

Although systemic chemotherapy is technically simpler than hepatic arterial infusion (HAI) chemotherapy, it has the disadvantages that the proportion of the drug reaching the intrahepatic lesion is low, and that the incidence of systemic adverse reactions is higher. Patients with HCC show lower tolerance to anticancer drug therapy because of impaired liver function. Pancytopenia may already be present due to concomitant cirrhosis, and marrow suppression is likely to occur with chemotherapy. Because of these features, hepatic arterial infusion chemotherapy is not commonly used in Europe and North America for HCC. However, in cases where the vital prognosis is determined by the intrahepatic lesion, control of the intrahepatic lesion may improve the prognosis, even if there are extrahepatic metastases. Therefore, hepatic arterial infusion chemotherapy is used for these cases in Japan. Hepatic arterial infusion chemotherapy requires certain skilled procedures, including catheterization, and is associated with a risk of vascular disorders related to catheter placement and reservoir management. On the other hand, it is a useful therapeutic modality, because it allows higher concentrations of the anticancer drug to be achieved in the target lesion. In fact, the drug is administered directly into the liver, allowing enhanced antitumor effect while being associated with a lower incidence of systemic adverse reactions. The proportion of CDDP into the hepatic tumor by first-pass kinetics was reported to be less than 5% after intravenous administration, but that of HAI administration was reported to be 48.4% (34%-55%)[18]. The response rate of CDDP monotherapy administered by HAI ranges from 14% to 42%[18-21] (Table 2). The dose-limiting toxicities (DLT) of CDDP are hematologic toxicity and nephrotoxicity, while hepatotoxicity is less significant. Therefore, it seems that a high therapeutic efficacy can be expected from selective HAI using a high concentration of CDDP.

In Japan, CDDP preparations for arterial infusion were approved in 2004. At variance with the conventional CDDP injection solutions (CDDP concentration, 0.5 mg/mL), the microfine powder CDDP preparation, whose average particle size lies between about 20 and 30 μm (IA-call®; NIPPON KAYAKU CO., LTD), is able to dispense an approximately 3-fold more concentrated CDDP solution than used in arterial infusion.

A multi-center phase-Ⅱ study of patients with unresectable advanced HCC was carried out in Japan[19]. In this study, where a highly concentrated CDDP solution (1.43 mg/mL) in warmed saline was used, the drug was administered by HAI. The dose was 65 mg/m^2 every 4-6 wk at each course, and 80 patients were given two courses.

Among the treated patients, 87.5% had underlying cirrhosis, and 48 patients had recurrent disease (46 of

Table 2 Hepatic arterial infusion chemotherapy

Author	Country and region	Publication year	Treatment schedule	*n*	RR (%)	PFS (M)	MST (M)	1yrs (%)	2yrs (%)	3yrs (%)	5yrs (%)
Court *et al*[18]	USA	2002	CDDP 50 mg/sq (+Radiation) q4w	67	37.0	-	10.7	-	-	-	-
Yoshikawa *et al*[19]	Japan	2008	CDDP 65 mg/sq q6-8w	80	33.8	-	-	67.5	50.8	-	-
Carr[20]	USA	2000	CDDP 125-200 mg/sq q4-8w	26	42.3	-	19.5	-	-	-	-
Chung *et al*[21]	Korea	2000	CDDP 2 mg/kg q8w	23	14.0	-	2.5	9	-	-	-
			CDDP 2 mg/kg q8w IFN-α 3MU. SC. 3/w	19	33.0	-	4.4	27	-	-	-
Patt *et al*[22]	USA	1994	CDDP 100 mg/sq, d1 DXR 30-35 mg/sq, d1 FUDR 60 mg/sq, d1-4 Leucovorin 15 mg/sq, d1-4	29	41.0	-	15.0	-	-	-	-
Toyoda *et al*[23]	Japan	1995	CDDP 5-10 mg/24h, d1-7 5-FU 500 mg/24h, d1-7	21	14.0	-	-	61.1	-	-	-
Okuda *et al*[24]	Japan	1999	CDDP 10 mg/1h, d1-5 5-FU 250 mg/5h, d1-5 q3-6w	31	70.9	-	-	-	-	45.7	45.7
Takayasu *et al*[25]	Japan	2000	EPI 30 mg/sq, d1, 6 CDDP 50 mg/sq, d2,7 ETP 60 mg/sq, d3, 4, 5	30	42.9	-	-	-	-	-	-
Tanaka *et al*[26]	Japan	2000	CDDP 10 mg/1h, d1-5 5-FU 250 mg/5h, d1-5 q4w	77	45.5	-	-	55.8	27.6	18.3	-
Ando *et al*[27]	Japan	2002	CDDP 7 mg/sq 1h, d1-5 5-FU 170 mg/sq 5h, d1-5 x4w	48	47.9	-	10.2	-	-	-	-
Kaneko *et al*[28]	Japan	2002	CDDP 75 mg/sq, d1, 15 5-FU 750 mg/sq, d1, 8, 15, 22 MTX 30 mg/sq, d1, 8, 15, 22 leucovorin 30 mg/sq, d1, 8, 15, 22 IFN-α-2b 3MU. SC, 3/w q4w	34	45.0	-	-	24	-	-	-
Sumie *et al*[29]	Japan	2003	CDDP 10 mg/1h, d1-5 5-FU 250 mg/5h, d1-5 x4w	16	56.3	-	32.4	-	-	-	-
Tanioka *et al*[30]	Japan	2003	CDDP 3 mg/sq 0.5 h, d1-7 5-FU 170 mg/sq continuous d1-7 x4w q5w	38	47.4	-	6.1	-	-	-	-
Lin *et al*[31]	Taiwan	2004	CDDP 10 mg/sq d1-5 MMC 2 mg/sq d1-5 leucovorin 15 mg/sq d1-5 5-FU 100 mg/sq continuous d1-5 x2w q3-4w	53	28.3	-	13.2	-	-	-	-
Yamasaki *et al*[32]	Japan	2005	CDDP 10 mg/body d1-5 5-FU 250 mg/body d1-5 leucovorin 12 mg(or isovorin 12.5 or 6.25 mg) d1-5	29	48.3	-	11.8	-	-	-	-
Nagai *et al*[33]	Japan	2007	CDDP 10 mg/h d1-5 leucovorin 12 mg/h d1-5 5-FU 250 mg/sq (4 h *vs* 22 h) x4w	37 (15 *vs* 22)	6.7 *vs* 31.8	-	7.4 *vs* 16.3	-	-	-	-
Park *et al*[34]	Korea	2007	5-FU 500 mg/sq d1-3 q4w CDDP 60 mg/sq d2	41	22.0	7	12.0	-	-	-	-

SC: Subcutaneous injection; CDDP: Cisplatin; DXR: Doxorubicin; EPI: Epirubicin; 5-FU: 5-fluorouracil; ETP: Etoposide; MTX: Methotrexate; FUDR: floxuridine; IFN: Interferon; RR: Response rate; PFS: Progression free survival; MST: Median survival time; 1yrs: 1 year survival rate.

these patients had a history of previous chemotherapy). The response rate was 33.8% (27/80) (95% CI: 23.6%-45.2%), and PR was achieved in these 27 patients with a median of 28.0 d (25-71 d). Multivariate analysis of the response rates revealed that the presence/absence of vascular invasion was a significant factor influencing the therapeutic effect, whereas no such relation was found for a history of previous chemotherapy. In regard to other anticancer drugs, response rates of 15.1%, 26.1% and 20.0% have been reported for arterial infusion monotherapy using epirubicin[35], mitoxantrone[36] and mitomycin C[37], respectively. Thus, CDDP showed higher antitumor efficacy than other anticancer drugs when administered by HAI. Multivariate analysis also showed that the prognosis was significantly poor in patients with vascular invasion, but the survival tended to be prolonged in patients who responded to the therapy. In regard to the incidence of grade 3 or more severe adverse events, anorexia occurred in 22.5% of the patients, vomiting in 6.3%, and abdominal pain in 1.3%, and all of these tended to improve within 1 wk. Grade 3 or more severe laboratory abnormalities included thrombocytopenia (25.0%), neutropenia (13.0%), leukopenia (1.3%), hypochromia (1.3%), and AST elevation (32.5%).

Abnormal values were usually found within 1 wk after the drug administration, and were almost completely back to pretreatment levels 2 wk later.

In this study, the incidence of gastrointestinal symptoms, such as anorexia and vomiting, and hematologic toxicities, such as leucopenia, thrombocytopenia and hypochromia, following arterial infusion of CDDP were similar to those observed after intravenous administration. Nephropathy was milder than after intravenous infusion of CDDP. Although liver damage occurred at a rather high frequency, it never resulted in death. The higher concentrations of the drug in the non-cancerous lesions caused by HAI were at the origin of the liver toxicity.

Concerning the multidrug regimens containing CDDP for HAI therapy, low-dose CDDP combined with 5-FU (low-dose FP) has been intensively investigated in recent years[23,24,26,27,29,30,32,33] (Table 2). A response rate of about 40% and a median survival time of 6-12 mo have been reported for this treatment. However, the optimal administration time of 5-FU each day, the number of treatment cycles, and the modalities of the maintenance therapy, i.e. three factors constituting standard treatment, have not been established yet. In addition, low-dose FP is characterized by a problem represented by the prolonged hospitalization due to the long duration of treatment. Park *et al*[34] administered FP therapy using a three-day treatment schedule, and reported favorable results with a median survival time of 12 mo. This schedule allows the treatment administration with a short-term hospitalization, and is therefore considered as cost effective.

Combined regimens containing three or more drugs, including the anticancer drug anthracycline, have also been studied. The response rates for these regimens have been reported to be in the range of 28%-45%[22,25,28,31] (Table 2).

EXPECTATIONS FOR CDDP USE *VIA* TRANSCATHETER HEPATIC ARTERIAL CHEMOEMBOLIZATION

Transcatheter hepatic arterial chemoembolization (TACE) is a therapeutic modality that comprises a combination of hepatic arterial infusion and embolization. Some recent reports concluded that TACE impacted on the survival rate of HCC patients[38,39]. Anthracyclines are commonly used for TACE, and these agents are usually mixed with lipiodol (lipiodolization).

The antitumor effect of lipiodolization has not been validated because necrotic areas in the diagnostic images have been dealt with according to different standards among the studies. However, combined regimens containing CDDP and lipiodol have been reported to yield response rates of 15%-57%, while corresponding rates of 45%-73% have been reported for the treatment combined with embolization using gelatin sponge particles. Thus, the response rates of TACE tend to be higher than other chemotherapies[5,6,20,40-50] (Table 3). Two relevant randomized controlled studies have been reported. A study, conducted by a French group and published in 1995[51], reported that the 1-year and 2-year survival rates following this therapy were better than those following conservative treatment, while no significant difference in the overall survival was observed. However, Lo *et al*[39] reported a significantly better overall survival despite using a relatively low dose of CDDP (median 10 mg/20 mL) (Table 4). This difference may be explained by the improved catheter management and other technical advances related to the vascular route of dosing.

In Japan, arterial infusion of micropulverized CDDP directly suspended in lipiodol has been investigated since the 1980s. Fundamental studies of CDDP/Lipiodol suspensions (lipiodol platinum suspension, LPS) have confirmed the extended-release nature of CDDP[44,52-56]. It is also considered that the lipiodol suspension prevents inactivation by protein binding of the drug, allowing a higher concentration of the drug to be maintained inside the tumor. LPS can be prepared conveniently if micropulverized preparations are used. LPS is prepared by directly mixing micropulverized CDDP with lipiodol (10-20 mg/mL), followed by adequate stirring until the powder is evenly dispersed. In regard to the precautions that must be followed for administration, LPS should be infused slowly into the hepatic artery *via* a microcatheter, without mixing with physiological saline. Since a fine powder is infused directly, occlusion is likely to occur in the infused blood vessels, and caution related to the infusion volume is necessary whenever TACE is employed. Similarly to the usual intravenous administration, hydration is necessary as a prophylaxis against nephropathy. Adverse reactions may be tolerable when pretreatment fluid infusion is employed. Therefore, TACE with LPS is considered to improve therapeutic results in advanced HCC patients. As for the usefulness of TACE using LPS, randomized controlled studies are warranted to compare with anthracyclines.

FUTURE PERSPECTIVES OF HEPATIC ARTERIAL INFUSION CHEMOTHERAPY

The advent of CDDP has resulted in a certain level of therapeutic efficacy of HAI. However, the response rate is still inadequate. Further studies of the optimal dosing schedule and optimal combinations, including new drugs, are awaited.

The treatment modality for HCC is determined by the stage of cancer and the hepatic functional reserve. It is also important not to cause reduction of the hepatic reserve during treatment, because this is a critical prognostic factor. Therefore, combined use of supportive treatment with liver-protective drugs, ramified amino acids, or other agents that improve or maintain hepatic function should also be considered.

In Asian countries, it has been speculated that HCC

Table 3 Transcatheter arterial chemoembolization (inc. transcatheter arterial infusion with lipiodol)

Author	Country	Publication year	Treatment schedule	Root	*n*	RR (%)	MST (M)	1yrs (%)	2yrs (%)	3yrs (%)	5yrs (%)
Ikeda *et al*[40]	Japan	1992	DXR/Lip 10.5 mg[1] MMC/Lip 7.8 mg[1] Lip 3.7 mL[1] CDDP 135.7 mg[1]	Lip-TAI	76	23.7	-	68.0	41.0	24.0	-
Yodono *et al*[41]	Japan	1992	CDDP 20 mg/sq, d1-5 ETP 30-40 mg/sq, d1-5 5-FU 250 mg/body, d1-26 + CDDP/Lip + GS	TAI +Lip-TACE	14	46.2	27.6	50.0	43.0	34.0	-
			CDDP 50 mg/sq, d2,8 ETP 50-60 mg/sq, d4-6 DXR 20 mg/sq, d1,7 + CDDP/Lip + GS	TAI +Lip-TACE	31	48.4	21.7	77.0	42.0	-	-
Hatanaka *et al*[42]	Japan	1995	CDDP 50-100 mg DXR 20-40 mg FUDR 3-5 g + GS	TACE	60	-	-	80.4	65.2	48.6	-
			CDDP 50-100 mg DXR 20-40 mg Lip 4.8 mL[1] FUDR 3-5 g + GS	Lip-TACE	78	-	-	86.3	55.3	34.8	-
			CDDP 50-100 mg DXR 20-40 mg Lip 4.9 mL[1] FUDR 3-5 g	Lip-TAI	159	-	-	65.9	50.3	36.2	-
Raoul *et al*[43]	France	1997	CDDP 70 mg (saline 140 mL)/Lip 10 mL + GS	Lip-TACE	64	57.0	-	42.2	22.1	2.8	-
Carr[20]	USA	2002	CDDP 125-200 mg/sq q4-8w + GS	TACE	31	58.1	30.7	-	-	-	-
Shibata *et al*[44]	Japan	1989	CDDP 20-150 mg (CDDP/Lip: 20 mg/mL)	Lip-TAI	71	46.5	-	55.0	-	-	-
Kawakami *et al*[45]	Japan	1993	CDDP 50 mg (CDDP/Lip: 10 mg/mL)	Lip-TAI	12	12.5	7.0	-	-	-	-
			CDDP 50 mg (CDDP/Lip: 10 mg/mL) + GS	Lip-TACE	30	45.5	25	81.3	56.8	-	-
Ono *et al*[46]	Japan	2000	CDDP 50 mg[1] (CDDP/Lip: 10 mg/mL) + GS	Lip-TACE	38	45.0	-	-	49.0	-	19.0
			DXR 43 mg[1] (20-50 mg)/Lip + GS	Lip-TACE	46	38.0	-	-	31.0	-	6.0
Kamada *et al*[47]	Japan	2001	CDDP 41 mg[1] (15-70 mg)/Lip + GS (CDDP/Lip: 10 mg/mL)	Lip-TACE or Lip-TAI	108	15.0	24.0	81.0	-	41.0	19.0
			DXR 57mg[1] (20-100 mg)/Lip + GS	Lip-TACE or Lip-TAI	26	4.0	17.0	67.0	-	18.0	0.0
Maeda *et al*[48]	Japan	2003	CDDP 70.5 mg[1]/Lip (CDDP/Lip: 20 mg/mL)	Lip-TAI	143	57.3	-	89.2	65.3	48.8	29.6
			CDDP 78.4 mg[1]/Lip + GS (CDDP/Lip: 20 mg/mL)	Lip-TACE	96	62.5	-	85.2	67.0	48.7	24.2
Ikeda *et al*[5,6]	Japan	2009 -2004	CDDP 50 mg[1] (20-150 mg)/Lip (CDDP/Lip: 20 mg/mL)	Lip-TAI	94	51.1	30.0	81.6	65.2	39.8	18.3
			CDDP 70 mg[1] (30-150 mg)/Lip + GS (CDDP/Lip: 20mg/mL)	Lip-TACE	74	73.0	37.2	87.8	-	52.2	25.0
Uyama *et al*[49]	Japan	2008	CDDP80 mg[1] /Lip (40-100mg) + GS (CDDP/Lip: 20 mg/mL)	Lip-TACE	24	45.8	-	-	-	-	-
Yamashita *et al*[50]	Japan	2009	CDDP 35 mg/sq (CDDP/Lip: 10-20 mg/mL)	Lip-TAI	35	57.1	-	-	-	-	-

CDDP: Cisplatin, DXR: Doxorubicin; EPI: Epirubicin; ETP: Etoposide; 5-FU: 5-fluorouracil; FUDR: Floxuridine; MMC: Mitomycin C; GS: Gelatin sponge; Lip: Lipiodol; TAI: Trasctheter arterial infusion; TACE: Trasctheter arterial chemoembolization; RR: Response rate; MST: Median survival time; 1yrs: 1 year survival rate. [1]Mean.

Table 4 Randomized control trial (TACE)

Author	Country and region	Publication year	Treatment schedule	Root	*n*	RR (%)	1yrs (%)	2yrs (%)	3yrs (%)
Group d'Etude et de Traitment du Carcinome Hepatocellulaire[51]	France	1995	CDDP/Lip (70 mg/10 mL) + GS	Lip-TACE	50	16.3	62.0	37.8	-
			Conservative management	-	46	5.0	43.5	26.0	-
Lo *et al*[39]	Hong Kong	2002	CDDP/saline + Lip (1:1), (median: 10 mg/20 mL/body max: 30 mg/60 mL/body) + GS	Lip-TACE	40	39.0	57.0	31.0	26.0
			conservative management	-	39	6.0	32.0	11.0	3.0

CDDP: Cisplatin; GS: Gelatin sponge; Lip: Lipiodol; TACE: Trasctheter arterial chemoembolization; RR: Response rate; MST: Median survival time; 1yrs: 1 year survival rate.

mostly arises from mutations induced by hepatitis B virus and cirrhosis due to persistent infection with hepatitis C virus. Therefore, when these viruses are present, most patients will have recurrent disease within several

years, even if the disease is detected at an early stage and treated by resection or radical local treatment (e.g. RFA). In cases of early recurrence, i.e. within 2 years from radical treatment, it is highly likely that minute cancer cells were already present at the time of the previous treatment, leading to intrahepatic metastases or multinodular disease[57]. Therefore, to prevent early recurrence after hepatectomy or radical local treatment, we can anticipate a role of hepatic arterial infusion chemotherapy as adjuvant therapy. In addition, control of the hepatitis viral infection and prevention of carcinogenesis by interferon therapy are considered important factors to prevent recurrence occurring more than two years after the radical treatment[58].

Currently, the efficacy of anti-cancer drugs, as evaluated by the sensitivity of individual cancers, is being evaluated. Subsequently, tailor-made treatments for HCC will rapidly become available.

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GUIDELINES FOR BASIC SCIENCE

Activins and follistatins: Emerging roles in liver physiology and cancer

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Abstract

Activins are secreted proteins belonging to the TGF-β family of signaling molecules. Activin signals are crucial for differentiation and regulation of cell proliferation and apoptosis in multiple tissues. Signal transduction by activins relies mainly on the Smad pathway, although the importance of crosstalk with additional pathways is increasingly being recognized. Activin signals are kept in balance by antagonists at multiple levels of the signaling cascade. Among these, follistatin and FLRG, two members of the emerging family of follistatin-like proteins, can bind secreted activins with high affinity, thereby blocking their access to cell surface-anchored activin receptors. In the liver, activin A is a major negative regulator of hepatocyte proliferation and can induce apoptosis. The functions of other activins expressed by hepatocytes have yet to be more clearly defined. Deregulated expression of activins and follistatin has been implicated in hepatic diseases including inflammation, fibrosis, liver failure and primary cancer. In particular, increased follistatin levels have been found in the circulation and in the tumor tissue of patients suffering from hepatocellular carcinoma as well as in animal models of liver cancer. It has been argued that up-regulation of follistatin protects neoplastic hepatocytes from activin-mediated growth inhibition and apoptosis. The use of follistatin as biomarker for liver tumor development is impeded, however, due to the presence of elevated follistatin levels already during preceding stages of liver disease. The current article summarizes our evolving understanding of the multi-faceted activities of activins and follistatins in liver physiology and cancer.

Key words: Activin; Inhibin; Follistatin; Follistatin-like protein; Transforming growth factor β; Liver cancer

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INTRODUCTION

The activin family

Activins are cytokines belonging to the TGF-β family of growth and differentiation factors[1] and were named according to their first identification as activators of follicle-stimulating hormone (FSH) release from pituitary cells[2,3]. Like TGF-β, activins are formed *via* the covalent dimerization of two subunits[4]. So far, five different subunits participating in the formation of activins have been identified. The subunits activin beta A, beta B, beta

C and beta E were found in humans as well as other mammalian species, while activin beta D has only been identified in Xenopus laevis[5]. The four mammalian beta subunits are each encoded by a single gene, called INHBA, INHBB, INHBC and INHBE respectively[6]. INHBC and INHBE are closely linked in several species and are thought to have arisen from tandem duplication of an ancestral gene[7].

The different activin subunits can form homo- as well as heterodimers. A homodimer of two beta A subunits is called activin A, while a heterodimer of a beta A and a beta B subunit is called activin AB. The nomenclature for dimers of the other subunits follows the same scheme. While activins AB and AC have been described under physiological conditions *in vivo*[8,9], we and others have demonstrated the formation of activins AE, BC and CE after ectopic expression of the respective cDNAs in various cell lines[10-12]. Activin subunits are synthesized as pro-proteins of 350 to 426 amino acids[6]. The proteins are glycosylated in the pro-domain region, but addition of the carbohydrate group seems to be dispensable for secretion. This is in contrast to the related inhibin alpha subunit, a member of the TGF-β family and dimerization partner of activin subunits (see below)[13]. Dimers are created by intermolecular disulphide bond formation between the sixth of nine conserved cysteines in the mature proteins. The other cysteines are involved in the formation of intramolecular disulphide bonds, creating the so-called cysteine knot, typical for members of the TGF-β family and required for their biological activity[14].

Following dimerization, the protein is cleaved by pro-protein convertases of the subtilisin/kexin family in the ER and Golgi, producing a mature peptide chain of 115 or 116 amino acids. While the biologically active protein is secreted as a dimer of the mature peptides only, it has been suggested that the pro-region is required for correct folding, dimer formation and secretion[15]. Unprocessed, dimeric activin A was found to be biologically inactive[16]. Monomers have been reported to retain some affinity for the receptors of dimeric activin A but do not cause activation[17]. In addition to dimerization with another beta subunit, activin beta A and activin beta B can form heterodimers with the inhibin alpha subunit, giving rise to inhibins A and B, both inhibiting FSH release[18]. It remains uncertain if inhibin C exists, as there was evidence for the formation of a dimer between activin beta C and inhibin alpha in some[19] but not all reports[10].

Activin signal transduction

Like other members of the TGF-β family, activins are believed to signal *via* single-pass transmembrane receptors with an intracellular Ser-Thr kinase domain. This has been proven for activins A, B and AB. Activin A first binds to dimers of the type Ⅱ receptors ActR-Ⅱ (aka ACVR2) or ActR-ⅡB (aka ACVR2B), leading to the (preferential) recruitment and phosphorylation of dimers of the type I receptor ALK4 (aka ActR-IB/ACVR1B)[20]. While binding to the same type Ⅱ receptors, activins B and AB preferentially recruit ALK7 (ACVR1C) as type I receptor[21]. Upon ligand binding, receptors are typically internalized[22]. It has been questioned however, if this internalization is generally necessary for signal transduction[23]. As a consequence of activation, receptor-regulated Smads (R-Smads) are recruited to the receptor complex and phosphorylated by the type I receptor. This process is supported by accessory proteins like SARA and the motor protein kinesin-1. Depending on the identity of the receptor, either Smad 2 and Smad 3 (ALK4, ALK5, ALK7) or Smad 1, Smad 5 and Smad 8 (ALK1, ALK2, ALK3, ALK6) are recruited and activated[4]. For TGF-β it has been shown that the ligand can recruit different type I receptors, activating different subsets of Smads depending on the cell type[24]. So far, activins have only been shown to signal through Smad 2 and Smad 3[25]. R-Smads then form complexes with the common mediator Smad 4 and translocate to the nucleus where, together with cofactors, they are directly involved in regulation of gene expression.

In addition, recent evidence suggests Smad independent signaling of activin A *via* MAP kinases ERK 1/2 and p38[26] as well as the phosphatidylinositol 3'-kinase (PI3K)/Akt pathway[27]. Rho and JNK were also found to be stimulated by activin A[28].

ACTIVINS IN HEPATIC FUNCTION AND DYSFUNCTION

Beta A and beta B

Activin A represents the most extensively investigated activin. Multiple biological functions of activin A in a variety of cells and tissues have been described, including involvement in mesoderm induction[29], stem cell biology[30], reproductive biology[31], erythroid differentiation[32], systemic inflammation[33], cell death induction[34], wound healing[35], and fibrosis[36]. Knock-out mice for activin beta A show severe defects in craniofacial development and die shortly after birth[37]. Activin A potently inhibits mitogen-induced DNA synthesis in the liver and induces hepatocyte apoptosis *in vivo* and *in vitro*[38-40]. Activin beta A antisense oligonucleotides stimulated cell proliferation in the human hepatoma cell line HLF suggesting a growth inhibitory function of endogenous activin A[41]. In regenerating liver, activin beta A gene expression was reduced at time points when hepatocyte replication took place and was increased at time points when liver regeneration terminated[42]. Other studies, however, have described increased expression of beta A at earlier time points after partial hepatectomy[43,44].

Beside its effects on DNA synthesis and cell growth, activin A also regulates restoration of liver architecture after partial hepatectomy by stimulating collagen production in hepatic stellate cells (HSC) and tubulogenesis of sinusoidal endothelial cells[45,46]. Stimulation of HSC may also

contribute to liver fibrosis and several investigations have found elevated levels of activin beta A in fibrotic and cirrhotic rat livers[47-50]. In hepatocytes, activin A was also demonstrated to stimulate the expression of connective tissue growth factor (CCTF/CCN2), an important regulator of liver fibrosis[51]. Elevated levels of circulating activin A were found in patients with acute liver failure, chronic viral hepatitis, alcohol induced liver cirrhosis and hepatocellular carcinoma (HCC)[52-57]. Elevated serum activin A was also reported in a study with patients suffering from non-alcoholic fatty liver disease (NAFLD), with particularly high levels in the subgroup with non-alcoholic steatohepatitis (NASH)[58]. These patients also had an increased activin beta A/follistatin mRNA ratio in liver tissue. In the same study activin A was shown in Huh7 hepatoma cells to promote collagen Ⅲ and TGF-β 1 expression, matrix metalloproteinase (MMP) activity, induce mitochondrial beta-oxidation and down-regulate fatty acid synthase (FAS) activity. Together these findings suggest an involvement of activin A not only in fibrosis but also in lipid accumulation. A study from our group in contrast, has found reduced expression of activin beta A transcripts in tumor tissue from chemically-induced rat liver tumors[59]. In addition to a pro-apoptotic and a pro-fibrotic effect, activin A has also been linked to hepatic neoangiogenesis *via* stimulation of VEGF expression in human hepatoma cells[60]. With respect to hepatic differentiation, it has been shown that a gradient of activin/TGF-β signaling controls differentiation of hepatoblasts into hepatocytes and biliary cells in the mouse, with high signaling activity required for development into biliary cells[61]. The contributing activin/TGF-β ligands, however, have not been fully identified. Several studies have used activin A as part of protocols to differentiate human embryonic stem cells (hESC) into hepatocyte-like cells[62-65].

Like activin beta A, the beta B subunit is expressed in multiple tissues and organs[11,66]. Knock-out mice for beta B are viable but show defects in eyelid development and female reproduction[67]. When the coding region of the mature peptide of the beta A subunit gene was replaced with the corresponding region of the beta B subunit, the developmental defects of the beta A knock-out mice were only partially rescued indicating differences in receptor activation or downstream signals[68]. In the liver, the function of the beta B subunit is not well characterized. One reason for this might be the low expression level in normal rat liver, where we observed the beta B subunit to be the only activin subunit undetectable by RNAse protection assay[11]. By immunohistochemistry, however, weak staining of beta B was detected in hepatocytes of normal rat livers and in connective tissue septa in fibrotic livers[47]. Activin beta B mRNA was induced in stellate cells of CCl_4 treated rat livers[47] and exposure to the peroxisome proliferator di-n-butyl phthalate led to a transient surge of beta B mRNA expression 6 h after treatment[69]. With respect to biological activities, recombinant activin B, in contrast to activins A and AB, did not inhibit EGF induced DNA synthesis in primary rat hepatocytes[70]. In contrast to the rat, beta A and beta B transcripts are expressed to similar levels in human liver (Rodgarkia-Dara, unpublished observation). Ectopic expression of ALK7, the preferred type two receptor for activins B and AB induced apoptosis in hepatoma cell lines in a Smad and MAPK- dependent manner[71]. Both activin B and ALK7 have been linked to obesity and diabetes, two well-known risk factors for HCC, *via* participation in regulatory circuits in adipose tissue and the pancreas[72-74].

Beta C and beta E

In contrast to beta A and beta B, whose expression level is the highest in reproductive organs, the liver is the organ where the beta C and the beta E subunit reach by far their highest expression levels. The activin beta C subunit was cloned from liver cDNA and demonstrated to be predominantly expressed in hepatocytes by Northern blot analysis and RNAse protection assays[11,44,75,76]. By immunohistochemistry, significant activin beta C expression has been detected in cells from additional organs, including the prostate, ovary, testes, and pituitary gland[10,77]. After partial hepatectomy, a transient down-regulation of activin beta C expression was observed by several studies[42,44,78,79]. We have found reduced activin beta C expression in HepG2 and Hep3B hepatoma cells versus normal liver tissue[80] and a drop of beta C expression was also described in rat hepatocytes during primary culture with and without EGF treatment[44]. In contrast, increased activin beta C expression was reported in rat liver during the development of CCl_4-induced cirrhosis[48,81] and in response to treatment with the peroxisome proliferator bi-n-butyl phthalate[69]. The functions of the activin beta C subunit are controversial. Activin beta C knock-out mice developed normally and liver regeneration after partial hepatectomy proceeded similar in knock-out animals and wild-type littermates[82]. Studies from our group showed that ectopic expression of activin beta C induced apoptosis in human (HepG2, Hep3B) and rat (H4IIEC3) hepatoma cells and delayed liver regeneration in mice[80,83]. In contrast, in AML12 cells, an immortalized mouse hepatocyte cell line, and in primary rat hepatocytes activin beta C increased DNA synthesis[84]. Adenovirus-mediated expression of activin beta C accelerated liver regeneration after partial hepatectomy in rats[85] and association of activin beta C immunoreactivity with mitotic hepatocytes was observed in regenerating liver after partial hepatectomy[42]. Activin C does not activate activin A-responsive promoters and it was suggested that the beta C subunit down-regulates the levels of bioactive activin A *via* the formation of signaling-incompetent activin AC heterodimers in PC3 human prostate cancer cells[9,86]. In a recent study from the same group, it was shown that homodimeric activin C inhibited activin A-induced Smad2 phosphorylation and growth inhibition, and that activin beta C transgenic mice develop prostate, testis and liver pathologies

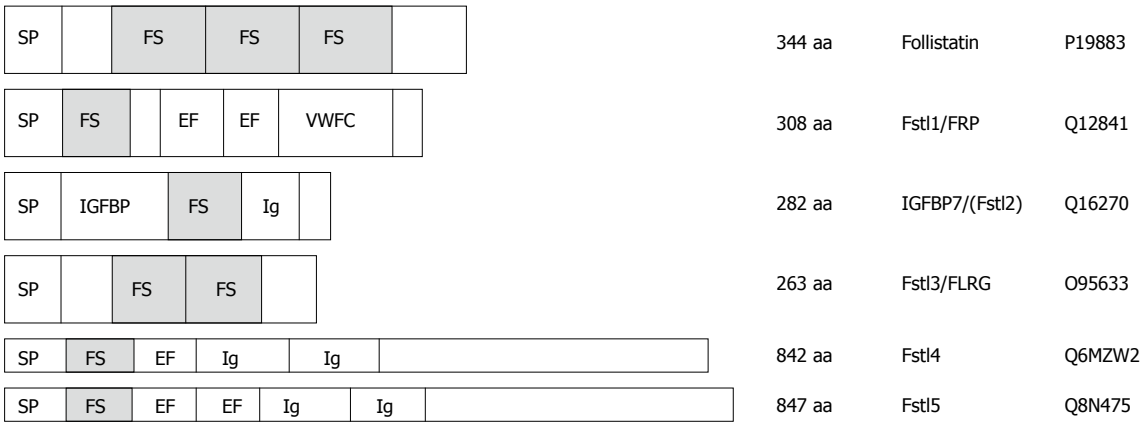

Figure 1 Number and arrangement of follistatin/Kazal-like domains in follistatin-like proteins. SP: Signal peptide; FS: Follistatin/Kazal-like domain; EF: EF-hand domain; VWFC: von Willebrand factor type C repeat; IGFBP: IGF-binding protein N-terminal domain; Ig: Immunoglobulin-like domain. Text to the right shows the number of amino acids (aa), most common name(s) and Uniprot accession number.

suggestive of an activin A antagonistic effect[87]. In line with these observations, elevated beta C immunoreactivity was found in human prostate, testis and liver cancers[87].

Like beta C, also the beta E subunit is highly expressed in the liver, but has been detected at lower levels in several other tissues as well[7,11,88,89]. In the liver of the developing mouse, activin beta E expression could not be detected until the very late stages of embryonic development and peaked at birth[82]. The biological functions and molecular interaction partners of activin beta E remain largely unknown. Like beta C, beta E knock-out (as well as beta C, beta E double knock-out) mice developed normally and showed no impairment of liver function or regeneration[82]. In vitro, overexpression of activin beta E in the human hepatoma cell lines HepG2 and Hep3B, as well as in the murine hepatocyte cell line AML12, caused decreased proliferation and induced apoptosis[12,80]. *In vivo*, transient overexpression of activin beta E inhibited regenerative DNA synthesis in mouse liver[83], while mice constitutively overexpressing the protein showed impaired growth of pancreatic exocrine cells[90]. Following partial hepatectomy, activin beta E mRNA increased rapidly and decreased to near-basal levels after 48 h[82]. We observed a diurnal variation of beta E mRNA depending on food consumption in the rat liver and a surge of beta E expression in response to bacterial lipopolysaccharide (LPS) stimulation was also described[1,89]. Additionally, beta E expression was found and confirmed to be elevated in HepG2 cells as a consequence of phospholipidosis, a lipid storage disorder[91,92]. Expression of activin beta E was also significantly increased in the lung following airway inflammation[93] and in brains of rats infected with Borna disease virus[94]. Interestingly, a neuronal component has also been implied by recent work describing reduced anxiety-related behavior in mice overexpressing activin beta E[95]. Overexpression of the tumor suppressor RASSF1A stimulated expression of beta E, while knock-down of endogenous RASSF1A in nasopharyngeal epithelial cells resulted in beta E downregulation[96]. Finally, in gene chip analysis, mRNA levels from INHBE were found to be altered in HepG2 in response to hypoxia[97]. One possible mode of action for activin beta E was described by Chow *et al*[96], who demonstrated that the expression of Inhibitor of DNA binding 2 (Id2) protein is down-regulated in response to overexpression of activin beta E. Id2 is a known target of TGF-β and a potential oncogene[98]. Large scale analysis identified mutations in the INHBE gene in breast cancer[99]. An evaluation of single nucleotide polymorphisms (SNPs) in genes coding for activins in testicular cancer showed a correlation for the risk of disease and mutations in INHBA but not in INHBB, INHBC or INHBE[100].

FOLLISTATINS AND THEIR ROLE IN ACTIVIN ANTAGONISM AND LIVER DISEASE

Follistatin was discovered as antagonist of activin activity with respect to FSH release from pituitary cells[101]. Sequence analysis of follistatin revealed no homology to the TGF-β family, but the presence of three domains with a similar architecture, namely 10 cysteines spaced in a conserved fashion resulting in a characteristic pattern of intramolecular disulphide bond formation[102]. Accordingly, this domain was termed follistatin domain and bears resemblance to the Kazal domain of serine proteinase inhibitors. Follistatin domains have been identified in a number of additional extracellular proteins and some of these have been filed as follistatin-related proteins or follistatin-like proteins[103-105] (Figure 1). The connection of follistatin and follistatin-like proteins with activin signaling and their involvement in hepatic functions is discussed below. Additional regulation of activin signal transduction takes place at the receptor level by co-receptors, such as cripto, nodal, betaglycan, or BAMBI and intracellularly, for instance by the inhibitory Smad 6 and 7, and has been reviewed elsewhere[106,107].

Follistatin

The biological activities described for follistatin seem to depend largely on its interaction with activins and other members of the TGF-β family. Follistatin, which is expressed in most organs expressing activins[66,108], binds mature secreted activin A with high affinity[109-111]. Complex formation with follistatin completely abolished receptor binding of activin A, thus blocking activin signaling[110,112]. Two follistatin molecules embrace one activin dimer and bury one-third of its residues and its receptor binding sites[113]. Alternative splicing and protein processing of a single follistatin gene results in secretion of three major isoforms containing 288, 303, and 315 amino acids[109]. Of the three follistatin domains present in all follistatin isoforms[114], the first two, but not the third, are necessary for activin A binding[111,115]. Follistatin 288 binds to heparan sulfates, whereas this binding is blocked by an acidic tail in follistatin 315[109]. In addition to binding activins A, B, AB, and E, follistatin was also shown to bind and antagonize myostatin as well as BMPs 2, 4, 6, and 7[88,116-119]. Follistatin administration by intraportal infusion or adenovirus-mediated overexpression caused DNA synthesis and liver growth in the rat, presumably by antagonizing tonic inhibition of hepatocyte proliferation by activin A[120,121]. Following partial hepatectomy, follistatin expression was up-regulated after 24-48 h, the time period in which hepatocyte replication was increased[42]. Administration of exogenous follistatin after partial hepatectomy accelerated liver regeneration but led to impaired restoration of normal tissue architecture and compromised liver function[122-124]. Administration of follistatin in CCl_4-treated rats attenuated the formation of liver fibrosis[125]. These results likely reflect the ability of follistatin to antagonize both growth-inhibitory and pro-fibrotic activities of activin A.

In mouse and rat models of chemically induced liver tumors, we found follistatin expression to be up-regulated in about 60% of tumor tissue samples[59,126]. Moreover, we demonstrated that administration of follistatin stimulated DNA synthesis in preneoplastic rat hepatocytes in an *ex vivo* system, whereas hepatoma cell lines were unresponsive to exogenous follistatin possibly due to autocrine production of follistatin or other activin antagonists[59,126-128]. Knock-out mice for parkin, an E3 ubiquitin ligase implicated in Parkinson's disease and frequently deleted in HCC and hepatoma cell lines[129], develop liver tumors in a follistatin upregulation-dependent fashion[130]. In human HCC elevated follistatin levels were found in the tumor tissue and the circulation of patients[53,59,131]. However, follistatin had no benefit as surveillance biomarker for HCC development in patients with alcoholic and non-alcoholic liver disease (ALD and NAFLD) due to the already elevated levels in the underlying liver pathologies[131]. Whether or not, interaction of follistatin with TGF-β family members other than activins (myostatin, BMP, GDFs) or with angiogenin[132] plays a role in liver tumorigenesis remains to be explored.

Follistatin-like proteins

Follistatin-like 1 (fstl1, also called follistatin-related protein, FRP or Tsc-36) contains only a single follistatin domain and no activin-binding activity has been reported. In fact, the interaction partners of fstl1 on a molecular level have not been identified and its function is far from clear. Fstl1 itself was identified as a TGF-β inducible gene[133] and has been implicated in inflammation and cardioprotection[134-136]. It has been suggested to act as a potential tumor suppressor in epithelial cancers[137-140] but is over-expressed in astrocytic brain tumors[141]. Considering hepatoma cells, we recently demonstrated that the expression of fstl1 is low in HepG2 cells, which show an epithelial morphology/proteome pattern and high in Hep3B cells with fibroblastoid characteristics. These observations suggest fstl1 as potential indicator of epithelial-mesenchymal transition (EMT)[142].

The term follistatin-like 2 (fstl2) is only rarely used. It refers to a protein described to have IGF- (insulin-like growth factor) as well as activin-binding activity and sequence homology with follistatin[143,144]. This protein was also termed mac25 and angiomodulin but is better known as IGFBP7 (IGF binding protein 7) or IGFBP-rP1 (IGF binding protein-related protein 1)[145]. It has been suggested to act as tumor suppressor, because its expression is reduced in neoplastic tissues of different cancer types including liver tumors from SV40T/t antigen transgenic mice[146]. However, the biological relevance of IGFBP7 binding to activin is still unclear. In the course of evolution fish went through whole genome duplication and the term fstl2 has also been used (synonymously with fstl1b) to denote the second zebrafish orthologue of mammalian fstl1.

Among the follistatin like proteins follistatin-like 3 (fstl3), encoded by follistatin-related gene (FLRG), has the highest overall similarity with follistatin and shares its ability to bind TGF-β family proteins, but contains only two instead of three follistatin domains[147]. The FLRG gene was originally identified as a target of chromosomal rearrangement in leukemia[148]. The highest tissue expression of FLRG was found in placenta, whereas highest follistatin expression was found in ovary, testis, and pituitary[147,149]. In HepG2 hepatoma cells, expression of both FLRG and follistatin was induced in response to activin A treatment suggesting that they participate in a feedback loop to restrict activin A signals[150]. FLRG knock-out mice developed increased pancreatic islet number and size, beta cell hyperplasia, decreased visceral fat mass, and hepatic steatosis. This is in line with a physiological role of fstl3 in antagonizing activin and myostatin activity in the pancreas, adipose tissue and liver[151]. Elevated expression of FLRG has been linked to breast cancer[152] and we have found increased FLRG transcript levels in chemically induced rat liver tumors but not in human liver tumor specimens[59].

Recently follistatin-like 4 and 5 were identified as two additional follistatin-related proteins[153], but their expression pattern and function have yet to be worked

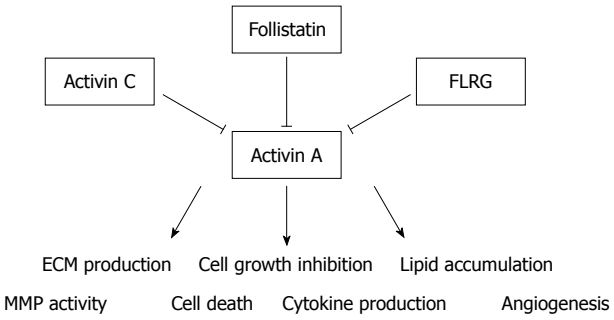

Figure 2 Reported effects of activin A on liver cells and inhibition by activin and follistatin family members. A potential function of the other family members, as agonists or antagonists of hepatic activin signal transduction or as regulators of activin A-independent activities in the liver, remains to be elucidated. ECM: Extracellular matrix; MMP: Matrix metalloproteinase.

out. In addition to the follistatin-like proteins, SPARC, agrin, tomoregulin and others contain one or more follistatin domains, but none of these have so far been connected to activin signal transduction[103,104].

CONCLUSION

Despite the apparent gaps in our knowledge, it is becoming increasingly clear that tightly regulated activin signals are of fundamental importance for the maintenance of liver architecture and cellular homeostasis. While still much has to be learned, especially about the less explored members of the activin and follistatin families, the pace of progress has appreciably sped up in recent years. Deregulated expression of activin A and/or follistatin has been consistently observed in liver cancer in human patients and in a growing number of animal models, and was shown to causally contribute to the inflammatory and fibrotic conditions that promote carcinogenesis (Figure 2). The picture that emerges is that inflammation-associated elevated activin A levels contribute to fibrotic tissue remodelling and cell death of normal hepatocytes, whereas preneoplastic and neoplastic hepatocytes become resistant to activin A-induced growth control, at least in part through overexpression of follistatin. Conditional and liver cell type-specific knock-out of activin beta A and follistatin in mouse hepatocarcinogenesis models could shed further light on the contribution of the activin-follistatin axis to liver cancer development. For the two activin subunits with predominant expression in hepatocytes, namely beta C and beta E, as well as for fstl1, 4 and 5 future efforts should be directed at elucidating their molecular interaction with cell surface receptors or secreted proteins as a prerequisite to better understand their biological activities. Although the complexity of the system may sometimes seem daunting, the hope is well founded that in the not-too-far future, the increasing knowledge on activins and follistatins will translate into improved diagnostic or therapeutic opportunities for patients suffering from chronic liver disease and HCC.

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Proteinases and their inhibitors in liver cancer

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Abstract

Proteinases are known to be involved in many cancer-related processes, particularly in the breakdown of extracellular matrix barriers in the course of tumor invasion and metastasis. In this review we summarize the current knowledge about the role of the most important matrix-degrading proteinases (cathepsins, matrix metalloproteinases, plasmin/plasminogen activators) and their respective inhibitors in liver cancer progression and metastasis.

Key words: Cathepsin; Cystatin; Hepatocellular carcinoma; Metalloproteinase; Plasminogen activator; Tumor invasion; Metastasis

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INTRODUCTION

Liver cancer represents the seventh most frequent malignancy, as manifested by more than 50.000 new cases per year. This corresponds to 6% of all cancers diagnosed in the year 2000[1]. The most common primary liver tumors are hepatocellular carcinomas (HCCs). Although many improvements have been made in terms of diagnosis and treatment, HCCs are usually associated with poor clinical prognosis, with a mean life expectancy of less than 6 mo. Surgical resection is only possible in 10%-20% of incidences and cures less than 5% of the patients. Tumor recurrence as well as intrahepatic and vascular metastasis severely affect the clinical outcome of this disease[2]. Interestingly, HCCs develop mainly in chronically injured tissue and are frequently associated with liver fibrosis. As a consequence of the development of fibrosis, HCC cells are often embedded in a stroma rich in extracellular matrix (ECM) proteins, which may culminate in the formation of a capsule surrounding the cancerous tissue[3]. However, aggressive HCCs have the capacity to penetrate such ECM barriers and spread into the surrounding parenchyma, leading to intrahepatic metastasis and portal venous invasion[4].

Various proteinases appear to be involved in the breakdown of ECM components during tumor invasion and metastasis, including plasmin and plasminogen activators, matrix metalloproteinases (MMPs), and cathepsins[5-7]. It has been shown that the synthesis of matrix-degrading proteinases is frequently upregulated in tumors. Cancer cells can also increase the proteolytic load in their environment by mobilization of proteinases from intracellular stores, and by acquisition and activation of proteinases released by stromal cells[8]. The degree of local ECM proteolysis is regulated by the concomitant secretion of endogenous proteinase inhibitors. The intricate balance between individual proteinases and their respective inhibitors implies that invasive tumor cells precisely coordinate ECM proteolysis with other cellular events required for effective invasion, such as cell-matrix attachment, detachment and migration[9].

Hepatocytes produce only a limited array of protein-

Table 1 Liver proteinases implicated in tumor progression and metastasis

Name	Type	Inhibitors	Localization	Physiological function
Cathepsin B	Cysteine	Cystatins (A-E)	Lysosomal	General protein turnover
Cathepsin D	Aspartic	-	Lysosomal	General protein turnover
Cathepsin L	Cysteine	Cystatins (A-F)	Lysosomal	General protein turnover
MMP-2	Metallo	TIMPs (1-4)	Extracellular	Matrix remodelling
MMP-3	Metallo	TIMPs (1-4)	Extracellular	Matrix remodelling
MMP-7	Metallo	TIMPs (1-4)	Extracellular	Matrix remodelling
MMP-9	Metallo	TIMPs (1-4)	Extracellular	Matrix remodelling
uPA	Serine	PAI-1, PAI-2	Extracellular	Fibrinolysis
tPA	Serine	PAI-1	Extracellular	Fibrinolysis

ases with matrix-degrading potential under normal, quiescent conditions. Besides plasminogen, the constitutively expressed enzymes most relevant to ECM degradation are the lysosomal proteinases cathepsin B, cathepsin D and cathepsin L[10-12] (Table 1). Other important matrix-degrading proteinases such as matrix metalloproteinases (MMPs) as well as plasminogen activators are usually undetectable. However, it has been reported that fetal rat hepatocytes can be stimulated to synthesize a selected range of MMPs and plasminogen activators[13]. Furthermore, expression of certain MMPs and plasminogen activators is enhanced during liver regeneration[14,15]. Nevertheless, even in the regenerating liver, ECM proteolysis is a tightly controlled process due to the concomitantly increased synthesis of proteinase inhibitors[14].

In the following sections, we review the current knowledge about the relevance of the balance between cathepsins, matrix metalloproteinases, plasminogen activators and their respective inhibitors for HCC progression and metastasis.

LYSOSOMAL PROTEINASES (CATHEPSINS)

In recent years, significant progress has been made in the biochemical and structural characterization of lysosomal proteinases. It has been shown that these enzymes participate in physiological processes other than bulk proteolysis in the lysosomes. Three proteinases appear to be present in all mammalian lysosomes: the aspartic proteinase cathepsin D, and the cysteine proteinases cathepsin B and cathepsin L[16]. Lysosomal cysteine cathepsins belong to the papain superfamily of cysteine proteinases, whereas cathepsin D is closely related to the major digestive enzyme pepsin[17,18].

Cathepsins are usually delivered in their zymogen forms to lysosomes. The acidic internal milieu of these compartments then triggers the largely autocatalytic proteolytic maturation of the latent proenzymes[19]. The rate-limiting factor in lysosomal targeting is the capacity of the endogenous sorting receptors, which results in the secretion of varying amounts of newly-synthesized proteinase precursors[20]. Under normal circumstances, these secreted forms exhibit only insignificant proteolytic activity. However, it was shown that at least secreted procathepsin B can be seen as a latent enzyme pool, which, upon (auto)activation in the acidic microenvironment around tumor cells, may cause local proteolysis[21].

Cathepsins can promote tumor invasion in different ways: (1) by direct cleavage of ECM/basement membrane components; (2) by activation of other proteinases[22-24] which in turn degrade ECM components; or (3) by cleavage of cell adhesion proteins on the cell surface, thus initiating the disruption of intercellular junctions[25].

CYSTEINE CATHEPSINS AND LIVER CANCER

The human genome encodes 11 cysteine cathepsins (B, C, F, H, L, K, O, S, V, X and W), all structurally closely related to the prototypic plant cysteine proteinase papain[26]. Cysteine cathepsins are often upregulated in various human cancers, and have been implicated in distinct tumorigenic processes such as angiogenesis, proliferation, apoptosis and invasion[7,25]. Using cathepsin knock-out mice, various groups have recently provided strong evidence for distinct functions of individual cathepsins in tumor progression and metastasis[27-29]. To date, the lysosomal cysteine proteinase most thoroughly studied in the context of cancer is cathepsin B, which has been reported to promote tumorigenesis in multiple ways[27,29,30].

So far, very little is known about cysteine cathepsins in liver cancer. However, there is some evidence that cathepsin B (CB) contributes to the invasive potential of hepatoma cells. Early studies reported differences between the subcellular distributions of CB in highly invasive murine Hepa cl9 hepatoma cells and normal hepatocytes, with significantly more CB associated with non-lysosomal membranes/vesicles in the tumor cells. This was attributed to transformation-induced changes to intracellular CB trafficking[31], a hypothesis further substantiated by subsequent morphological studies. While the enzyme was found to be restricted to perinuclear (presumably lysosomal) vesicles in an embryonic liver cell line, it was detected in vesicles adjacent to the cell membrane and in localized regions (possibly caveolae) of the surface of Hepa cl9 cells[32]. Evidence for the association of CB with caveolae in

tumor cells has been provided[33]. Moreover, it was found that CB synthesis and activity is significantly higher in Hepa cl9 cells than in normal liver cells[34]. Hence, these findings support the notion that alterations in the expression and subcellular distribution of CB contribute to the invasiveness and the metastatic potential of HCCs.

A detailed analysis of the biosynthesis and intracellular transport of another cysteine cathepsin, cathepsin C, in rat Morris hepatoma 7777 cells also revealed unusual features[35]. This can be at least partially explained by the deficiency of these cells in the main lysosomal sorting receptor, the mannose 6-phosphate/insulin-like growth factor Ⅱ receptor (M6P/IGF2R), a protein frequently absent and/or mutated in HCCs[36-38]. Intracellular sorting of cathepsin C in Morris hepatoma 7777 cells appears to involve MPR46, the second mammalian M6P receptor[39]. However, there is also evidence for M6P-independent membrane association and lysosomal delivery of cathepsin C in these cells[40].

In healthy tissue, the endogenous activities of cysteine cathepsins are tightly regulated by specific protein inhibitors, the cystatins. Type I cystatins (stefins) are located in the cytosol, whereas type Ⅱ cystatins are secretory proteins[41]. Alterations to the balance between cysteine cathepsins and cystatins have been postulated to contribute to tumor growth and malignant progression in various cancers[42]. Indeed, ectopic expression of cystatin C has been shown to reduce the tumorigenic and invasive potential of cancer cells[43,44]. Conversely, genetic ablation of this cystatin accelerated angiogenesis and tumor proliferation in a pancreatic cancer model[28]. Only very few reports have dealt so far with the role of cystatins in liver cancer. In one study, no obvious differences were found between the subcellular localizations of stefin A, stefin B and cystatin C in murine Hep cl9 hepatoma and embryonic liver cells[32]. However, a unique membrane-associated form of stefin A has been isolated from Hep cl9 tumors[45]. An intriguing novel cystatin, cystatin F, was identified in a screen for genes associated with liver metastasis[46]. The subcellular localization of cystatin F is highly unusual since this proteinase inhibitor is delivered to endosomal and lysosomal compartments[47,48]. It remains to be established whether the presence of cystatin F in lysosomes relates rather to the pro- than anti-invasive activity of this cystatin in malignant tumor cells.

CATHEPSIN D AND LIVER CANCER

The aspartic proteinase most extensively investigated in the context of cancer is cathepsin D (CD), with a particular emphasis on its role in breast cancer[49]. Comparatively little information is available on the relevance of this proteinase for liver cancer. CD was found to display a higher activity in hepatoma tissue than in normal human liver tissue. Interestingly, this coincided with an elevated M6P content of hepatoma cathepsin D[50]. Furthermore, the secretion of CD was markedly elevated in M6P/IGF2R-deficient rat Morris hepatoma 7777 cells when compared with normal hepatocytes. These cell types also differed in their ability to process CD into its mature forms. Remarkably, intracellular retention of CD in Morris hepatoma 7777 cells was largely insensitive to treatment with lysosomotropic bases, which are known to perturb M6P-dependent transport to lysosomes[51]. A similar observation was made for M6/IGF2R-positive human HepG2 hepatoma cells[52], thus ruling out that this phenomenon is linked to the M6P/IGF2R status of the cells. For HepG2 cells, evidence has been provided that biosynthetic transport of CD to lysosomes can occur in a M6P-independent manner[53]. This could be at least partially due to the transient association of procathepsin D with prosaposin[54]. It has been shown that prosaposin can undergo lysosomal delivery in the absence of a functional M6P receptor system, possibly via interaction with sortilin[55,56].

MMPS, TIMPS AND LIVER CANCER

More than 25 human proteins and plenty of homologues from other species are known to make up the MMP (matrix metalloproteinase) family. MMPs are classified into five subgroups regarding their preferential degradation of different matrix substrates: interstitial collagenases, type Ⅳ collagenases/gelatinases, matrilysins, stromelysins and membrane-type MMPs (MT-MMPs). Most MMPs contain several conserved functional domains, including a catalytic domain containing a highly conserved zinc-binding site and a hemopexin-like domain involved in substrate recognition[57-59]. All MMPs are initially synthesized as latent precursors. Conversion into the respective active species requires proteolytic removal of the inhibitory prodomain by other MMPs, serine proteinases or cathepsins[24,60-62].

MMPs are suggested as key regulators of tumor growth and metastasis. Based on their enzymatic properties, the MMPs most relevant to tumor invasion and metastasis are the type Ⅳ collagenases/gelatinases. The most prominent gelatinases, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), are able to degrade type Ⅳ collagen and other components of the basement membrane, which is the first barrier tumor cells have to break through during metastatic dissemination[63]. Studies in transgenic mice have highlighted the importance of MMP-2 and MMP-9 for cancer progression and tumor invasion[64-66]. However, it should be pointed out that certain MMPs such as MMP-8 can also exhibit anti-invasive properties[67].

The biological activities of MMPs are controlled by TIMPs (tissue inhibitors of metalloproteinases), which act through the formation of a tight, noncovalent complex with their cognate enzymes. TIMP-1, TIMP-2 and TIMP-4 are soluble proteins, whereas TIMP-3 is membrane-bound[60,63].

Several MMPs have been implicated in liver cancer.

The induction/upregulation of various MMPs (e.g. MMP-2, MMP-3, MMP-7 and/or MMP-9) has been detected in tumorous liver tissue obtained from HCC patients[4,68,69]. Furthermore, synthesis of MMP-2 was observed in several malignant HCC cell lines, whereas their benign counterparts appear to lack this proteinase[70]. Moreover, the production of MMP-9 in transformed murine hepatocytes can be triggered by induction of epithelial-to-mesenchymal transition, concomitant with the acquisition of invasive properties[71]. Interestingly, hepatocyte growth factor (HGF) has been found to induce the synthesis of several MMPs in hepatoma cells. In particular, stromelysin-1 (MMP-3) became clearly detectable upon HGF stimulation of human HepG2 hepatoma cells. Intriguingly, invasion of HGF-treated HepG2 cells could be blocked by a synthetic MMP inhibitor as well as by antibodies to MMP-3. These results suggest that transformation-associated changes in MMP expression contribute to the invasive activity of malignant HCC cells[69].

Metastatic dissemination of tumor cells is also facilitated by reduced endogenous TIMP levels. It has been observed that the serum and tissue levels of hepatic TIMP-2 are significantly higher in HCC patients without metastasis than in those with metastatic disease. In the latter cases, both primary HCC tissues and intrahepatic metastases displayed low TIMP-2 levels. This qualifies TIMP-2 as an important prognostic factor in HCC patients[2].

It has been reported that antisense-mediated reduction of TIMP-1 accelerates tumor formation and disease progression in a mouse model of HCC. Conversely, ectopic overexpression of hepatic TIMP-1 interferes with oncogene-induced tumorigenesis. High TIMP-1 levels were found to inhibit tumor initiation as well as the progression to later stages in HCC development[72]. Using the same transgenic mouse strains, further studies revealed that TIMP-1 overexpression inhibits oncogene-induced hepatocarcinogenesis largely by reducing hepatocellular proliferation and tumor vascularization[73]. This was found to be due to the reduced levels of bioactive insulin-like growth factor Ⅱ (IGF-Ⅱ) in TIMP-1 overexpressing animals. It was postulated that the presence of ectopic TIMP-1 leads to reduced proteolysis of IGF-binding protein-3 (IGFBP-3) and thus elevated IGFBP-3 levels, which in turn lower the bioavailability of IGF-Ⅱ[74].

Collectively, these findings suggest that imbalances between MMPs and TIMPs may enhance the proteolytic load in HCC tissues and thus promote HCC progression and metastasis.

THE PLASMINOGEN ACTIVATING SYSTEM (UPA, UPAR, PAI-1) AND LIVER CANCER

Plasminogen activation plays an important role in tumor invasion and metastasis. This proteinase precursor circulates in the pericellular environment, waiting to be activated by proteolytic maturation. Plasminogen can be activated by either of two types of plasminogen activators: tissue-type plasminogen activator (tPA), or urokinase-type plasminogen activator (uPA). The precursor forms of tPA and uPA display significant enzymatic activity, but the catalytic efficiency of uPA is strongly increased by plasmin-mediated proteolytic processing[75,76]. The biological activities of uPA and tPA are controlled by two plasminogen activator inhibitors, PAI-1 and PAI-2. uPA is the enzyme of higher relevance for tumor biology, which is at least partially due to the occurrence of a cellular uPA receptor (uPAR). uPAR is a glycosylphosphatidylinositol (GPI)-anchored protein located at the cell surface where it binds uPA, which in turn interacts with plasminogen and activates the latter. In tumors, uPA is concentrated at focal adhesion points through association with uPAR, which is enriched in these regions. Thus, the highly specific ternary interaction of plasminogen, uPA and uPAR permits strictly regulated local proteolysis of ECM components at the contact sites between tumor cells and the basement membrane[77]. Studies in uPA-deficient mice have provided evidence for a decisive role of this proteinase in tumor progression and metastasis[78].

The expression of tPA in normal, quiescent liver is low or undetectable. However, stimulation of tPA synthesis is observed during hepatocyte proliferation[79]. Within normal quiescent liver tissue, uPA synthesis appears to be mainly due to the presence of non-parenchymal cells such as hepatic stellate cells and Kupffer cells. However, hepatocytes have the capacity to produce uPA when induced to proliferate[80]. In this context, it should be noted that uPA, via its capacity to trigger HGF activation[81], appears to play a crucial role in liver regeneration[82].

Interestingly, both plasminogen activators and uPAR are readily detectable in HCC tissues[83,84]. Among the components of the plasminogen activation system, uPA appears to be the most useful diagnostic indicator for intra-hepatic metastasis and a reliable prognostic factor for HCC recurrence[85]. Furthermore, the cumulative presence of uPA, uPAR and PAI-1 is a good predictor of HCC invasion and metastasis[84]. The cellular source of uPA and uPAR in HCC is still unresolved, since the expression of uPA and uPAR in HCC tissues appears to be largely confined to stromal and inflammatory cells[86]. However, human hepatoma cells produce uPA upon stimulation with HGF[87]. Furthermore, the invasiveness of HGF-treated HepG2 cells could be reduced by pharmacological uPA inhibition[88]. This suggests that uPA is a promising target for HCC therapy.

CONCLUSION

HCC is a severe and common disease all over the world. Novel drugs for HCC treatment are urgently needed. In the last decades, considerable information has been

gained about the role of matrix-degrading proteinases and their inhibitors in this disease. A number of proteinases and proteinase inhibitors have been identified as new markers for the prediction of HCC outcome. The available data suggest that synthetic proteinase inhibitors could be used to prevent HCC progression and metastasis. Given this knowledge, it appears possible that both HCC diagnosis and, hopefully, also its therapy, can be improved in the near future.

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GUIDELINES FOR BASIC SCIENCE

Multistep carcinogenesis of perihilar cholangiocarcinoma arising in the intrahepatic large bile ducts

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Abstract

Flat-type "biliary intraepithelial neoplasia (BilIN)" and papillary-type "intraductal papillary neoplasm of the bile duct (IPN-B)" are proposed as precursors of invasive, perihilar intrahepatic cholangiocarcinoma (ICC). Three carcinogenetic pathways are proposed: BilIN progressing to tubular adenocarcinoma, and IPN-B progressing to tubular adenocarcinoma or to colloid carcinoma. Carcinogenesis *via* BilIN was characterized by mucin core protein 2-/cytokeratin 20-(MUC2-/CK20-) with MUC1 expression, while carcinogenesis *via* IPN-B leading to tubular adenocarcinoma was associated with MUC1 expression or that to colloid carcinoma with MUC1-negativity. In both the BilIN and IPNB series, the expression of p21, p53, and cyclin D1 was upregulated with histological progression. Interestingly, p53 expression was upregulated at the invasive stage of BilIN, but was low in noninvasive BilIN, while p53 expression was upregulated in IPN-B1 and reached a plateau in IPN-B2 and invasive ICC. Expression of $p16^{INK4a}$, which was frequent in BilIN1, was decreased in BilIN-2/3 and invasive carcinoma. EZH2 expression showed a stepwise increase from BilIN to invasive carcinoma. Membranous expression of β-catenin and E-cadherin was more markedly decreased in ICC with BilIN than in ICC with IPNB. Interestingly, disruption of the membranous distribution of β-catenin and E-cadherin seems to result in the invasion and metastasis of carcinoma cells of BilIN and IPN-B expressing MMP-7 and MT1-MMP. Increased expression of cyclin D1 and c-myc was more frequent in the IPNB lineage than BilIN lineage, possibly related to the Wnt signaling pathway associated with the nuclear accumulation of β-catenin. In conclusion, BilIN and IPN-B progress to invasive ICC through characteristic multistep processes.

Key words: Intrahepatic cholangiocarcinoma; Biliary intraepithelial neoplasm; Intraductal papillary neoplasm; Mucin; Cytokeratin; Matrix metalloproteinase; Intrahepatic bile duct; Polycomb group protein

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INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is a highly malignant and progressive hepatobiliary carcinoma accounting for about 5%-15% of primary malignant liver tumors[1-3]. A majority of ICCs are classified as well to moderately differentiated adenocarcinoma, and once ICC shows invasive growth, it aggressively invades the surrounding tissue and is commonly associated with distant metastasis. Because it is often diagnosed at an advanced stage, ICC has a poor prognosis[2-4]. While

ICC usually arises in an apparently normal liver, chronic cholangitis such as hepatolithiasis and primary sclerosing cholangitis, occasionally precedes its development[3-5]. While the incidence of ICC is gradually increasing in the Eastern and Western world, the mechanism underlying the malignant progression is not well understood.

Recently, studies on hepatolithiasis have identified two distinct neoplastic biliary intraepithelial lesions preceding invasive ICC[3]. The first is a flat or micropapillary growth of atypical biliary epithelium recognizable under a microscope, which has been called "biliary dysplasia"[5], and corresponds to "biliary intraepithelial neoplasia (BilIN)", one of the precursor lesions of ICC in the World Health Organization's classification of tumors[6]. The other is grossly characterized by the prominent papillary growth of atypical biliary epithelium with distinct fibrovascular cores and not-infrequent mucin over-production, which was recently termed an intraductal papillary neoplasm of the bile duct (IPN-B)[7,8]. BilIN and IPNB are now regarded as biliary counterparts of pancreatic intraepithelial neoplasia and pancreatic IPMN[9,10].

There is increasing evidence that the development and progression of malignant tumors of the biliary tree and their precursor lesions are associated with multiple alterations of cell-regulatory genes such as oncogenes and anti-oncogenes[11]. Phenotypic changes such as mucin and cytokeratin expression have also been reported in ICC and its precursor lesions[12,13]. In this review, we will discuss the recent progress in studies on the precursor lesions of ICC with an emphasis on histopathologic changes and phenotypic expression, as well as molecular and genetic changes. For a better understanding of these issues, the anatomy of the biliary tree and clinicopathological classification of ICC will be briefly reviewed.

ANATOMY OF THE BILIARY TREE AND CLASSIFICATION OF ICC

Anatomy of the biliary tree

The biliary tree is divided into the intrahepatic, hilar (right and left hepatic ducts and their confluence), and extrahepatic bile ducts. The intrahepatic bile duct is a branch of the right or left hepatic duct, and the first to third branches of both hepatic ducts are known as the intrahepatic large bile ducts which are grossly visible. Intrahepatic small bile ducts are the branches of intrahepatic large bile ducts, and are classified into septal and interlobular bile ducts. Bile ductules are located at the periphery of the portal tracts[14].

Clinicopathological classification of ICC

ICC is usually divided into either the peripheral type or the perihilar type, according to the location of the tumor along the biliary tree[1,2,15,16]. Perihilar type ICC is presumed to arise from the intrahepatic large bile ducts, though the ones arising from both hepatic bile ducts and their confluence could also be included in this group when they are invasive. Peripheral ICC arises in the hepatic parenchyma. The growth pattern of ICC is also grossly classified as being mass-forming, infiltrating periductally, or growing intraductally[6,17]. A majority of ICCs are well to moderately differentiated adenocarcinomas, and, in addition, colloid carcinoma is also occasionally identifiable. A majority of perihilar ICCs show periductal infiltration or nodular type along the biliary tree, and to a lesser degree, intraductal growth, while almost all peripheral ICCs are of a mass-forming type.

HISTOPATHOLOGICAL FEATURES OF PRECURSOR LESIONS OF INVASIVE ICC ARISING IN LARGE BILE DUCTS: FLAT AND PAPILLARY LESIONS

It has recently become evident that ICCs undergo a multistep-carcinogenesis, and two types of precursor lesions of invasive ICCs have been proposed: a flat intraepithelial neoplasia (BilIN) (Figure 1A-D) and an intraductal papillary lesion (IPNB) (Figure 1E)[6,7,9,18,19]. While the former is identifiable under a microscope, the latter is identifiable on radiologic images or through macroscopic examination[6,7]. These two lesions usually occur in the large intrahepatic bile ducts, hilar bile ducts and extrahepatic bile ducts, are rarely present in the septal or interlobular bile ducts. In addition, BilIN and IPN-B occur more frequently during chronic inflammatory biliary diseases, such as hepatolithiasis, primary sclerosing cholangitis, infestation by liver flukes, as well as congenital biliary diseases[6-8,20]. When accompanied by invasive lesions, BilIN is known to progress to conventional ICC (tubular adenocarcinoma), whereas IPN-B is associated with colloid carcinoma (mucinous carcinoma) or conventional ICC (Figure 2)[7,8,21].

While the exact carcinogenetic processes *via* these two precursor lesions remains to be clarified, recent studies have shown that, during their carcinogenetic pathways, different phenotypic or genetic changes take place[14,18,22-25].

Characteristics of BilIN lineages

BilIN is histologically defined as a flat or micropapillary proliferation of atypical biliary epithelium, showing multilayering, piled-up nuclei, an increased nucleocytoplasmic ratio, a partial loss of nuclear polarity, and nuclear hyperchromasia and pleomorphism. BilINs are now classified three grades: BilIN-1 (corresponding to low-grade dysplasia), BilIN-2 (high-grade dysplasia), and BilIN-3 (carcinoma *in situ*) (Figure 1A-D)[9,19]. Briefly, BilIN-1 shows mild cellular/nuclear atypia such as nuclear membrane irregularity or nuclear enlargement with only a minimal disturbance of cellular polarity. BilIN-2 has evident cellular/nuclear atypia, but not enough to suggest an overt carcinoma, with a focal disturbance of cellular polarity. BilIN-3 shows a diffuse disturbance of cellular polarity with or without distinct cellular/nuclear atypia corresponding to an overt carcinoma, though there is no invasion beyond the basement membrane. In this review,

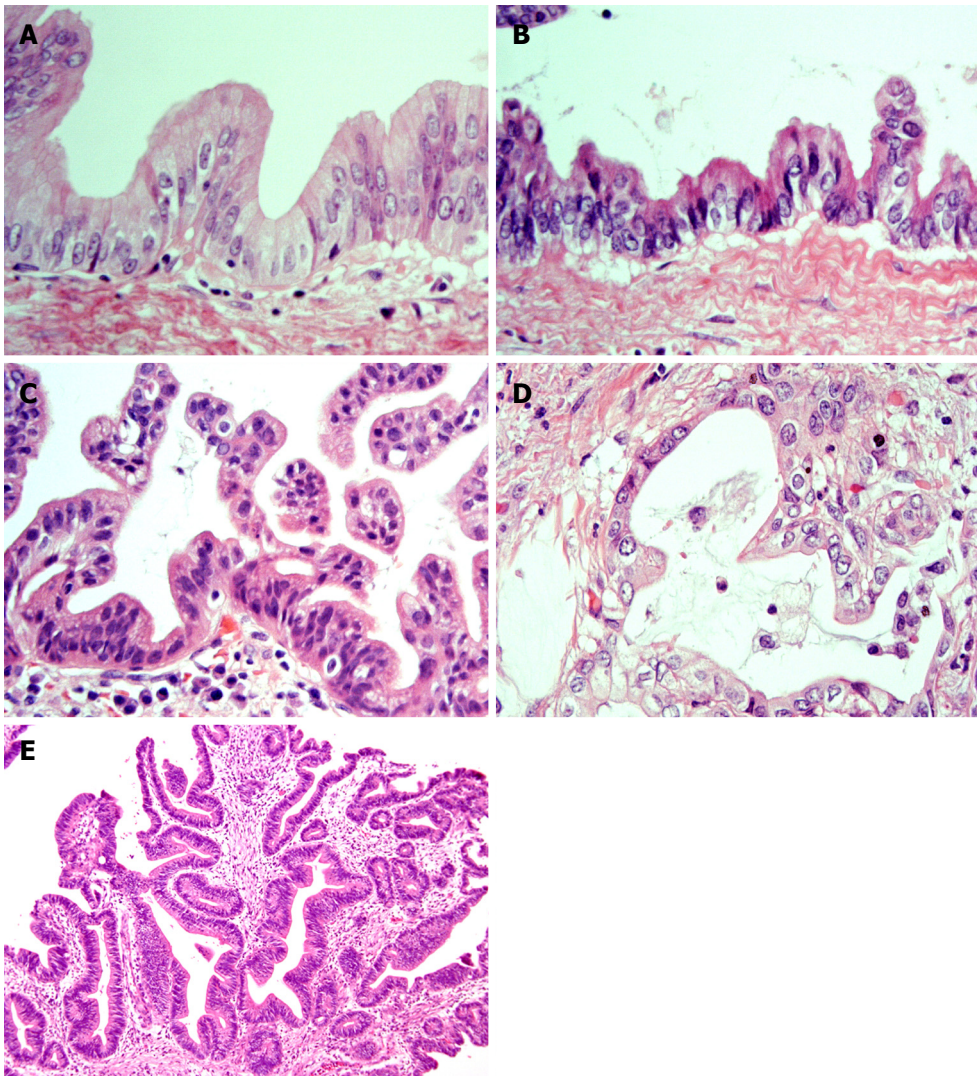

Figure 1 Representative histological features of neoplasms (HE staining). A: BilIN-1; B: BilIN-2; BilIN-3; D: Invasive ICC; E: IPN-B. BilIN: biliary intraepithelial neoplasm; ICC: intrahepatic cholangiocarcinoma; IPN-B: intraductal papillary neoplasm of the bile duct[22].

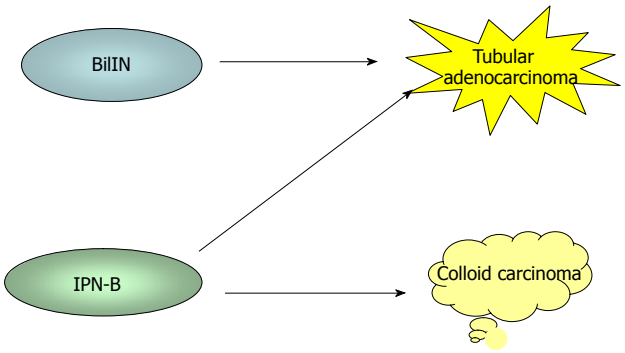

Figure 2 During biliary multistep carcinogenesis, BilIN is proposed to progresses to conventional ICC (tubular adenocarcinoma), whereas IPN-B is associated with colloid carcinoma (mucinous carcinoma) or conventional ICC.

BilIN-2 and BilIN-3 are grouped together as high-grade BilIN (BilIN-2/3). ICCs with BilIN are histologically tubular adenocarcinomas with micropapillary components, when present.

Characteristics of IPNB lineages

IPN-B has been defined as a prominent papillary proliferation of atypical biliary epithelium with distinct fibrovascular cores, showing nuclear stratification, piled-up nuclei, and nuclear enlargement (Figure 1E)[7,8,18]. Some parts show tubular components as well. IPN-B shows grossly visible papillary lesions in the saccular or cystically dilated bile ducts. All colloid carcinomas with IPN-B characteristically show macronodular lesions in contrast to the nodular growth or periductal infiltrative spread of tubular adenocarcinoma with BilIN or IPN-B. Because there are no well-established criteria for grading IPN-B, we graded IPN-B according to the World Health Organization's criteria for intraductal papillary mucinous neoplasm of the pancreas (IPMN-P)[26]. IPN-Bs were classified into two subgroups: IPN-B1 (corresponding to benign and borderline lesions of IPMN-P) and IPN-B2 (corresponding to carcinoma *in situ*). Interestingly, invasive IPN-B is of either the colloid type with variable amounts of tubular adenocarcinoma, or tubular adenocarcinoma without colloid components. Oncocytic papillary adenocarcinoma is also found in invasive areas. Recently, Shibahara *et al*[27] examined mucin-producing bile duct tumors, which are closely related to the IPN-B in this study, and reported that patients with tubular adenocarcinoma had a significantly lower rate of survival than those with mucinous (colloid) carcinoma.

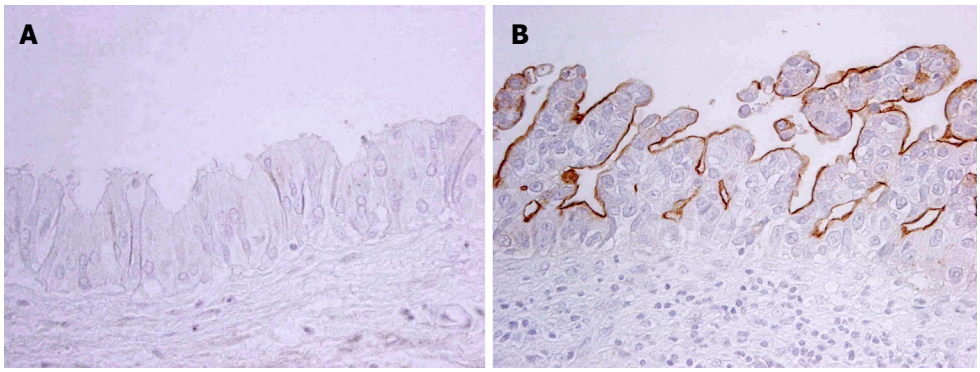

Figure 3 MUC1 is expressed in neoplastic cells of BilIN-3 (B) but not in those of BilIN1 (A) (Immunostaining). A: BilIN-1; B: BilIN-3.

EXPRESSION OF MUCIN CORE PROTEINS (MUCS) AND CYTOKERATINS (CKS) IN BILIN AND IPNB

Normal and pathologic intrahepatic bile ducts show a rather characteristic mucin and CK profile[10]. Physiologically, MUC3 and CK7 are expressed in the lining epithelium of biliary epithelial cells, though their expression is altered under pathologic conditions[14,28,29]. Interestingly, intestinal metaplasia (goblet cell metaplasia or Paneth cell metaplasia) is more frequent in cases of IPN-B than BilIN. In contrast, gastric metaplasia (foveolar or pseudopyloric gland metaplasia) was similarly observed in he BilIN and IPN-B lineages.

Expression of MUCs

The expression profile of MUCs in neoplastic biliary epithelia differed according to the pathways of the BilIN or IPN-B lineage[10]. MUC1, usually not detectable in non-neoplastic biliary epithelial cells, is expressed in one fourth of BilINs, especially in high-grade lesions (Figure 3), while it is only occasionally expressed in IPN-Bs. MUC1 is expressed in more than half of tumor cells in all ICC cases with BilIN, but is expressed in less than half of ICC cases with IPN-B. MUC5AC (gastric phenotype mucin) is frequently expressed in both BilIN lineages and IPN-B lineages in which foveolar (gastric) metaplasia is frequent. MUC2 is more commonly expressed in IPN-Bs and ICC with IPN-B while its expression is infrequent in BilINs and ICC with BilIN. Furthermore, in most cases of IPN-B, MUC2 was expressed in more than half of the neoplastic cells. Goblet cell (intestinal) metaplasia is frequently observed in IPN-B in which MUC2 expression (intestinal phenotype) is also frequent, whereas intestinal metaplasia is not frequent in BilIN lineage with no or infrequent MUC2 expression. These findings are compatible with the common occurrence of gastric metaplasia in both lineages, while intestinal metaplasia is more frequent in the IPN-B lineage.

As for the combined profile of MUC1 and MUC2 expression in each lesion, most IPN-Bs show the MUC1-/MUC2+ pattern[10,13,30]. Almost all colloid carcinomas exhibit the MUC1-/MUC2+ pattern. Tubular adenocarcinomas with IPN-B usually show MUC1+/MUC2+. These findings suggest that most non-invasive neoplastic lesions are MUC1-/MUC2+ during the carcinogenesis from IPN-B. When invasive ICC develops, a majority of tubular adenocarcinomas with IPN-B are MUC1-positive (MUC1+/MUC2+), whereas almost all colloid carcinomas retain a MUC1-negative pattern, such as MUC1-/MUC2+. In contrast, the MUC1-/MUC2- is most common in BilINs, and the frequency and degree of the MUC1+/MUC2- pattern increases stepwise in the BilIN lineage with a progression of histological grade (BilIN-1, BilIN-2/3 and ICC with BilIN). It is possible that the increased levels of MUC1 in BilIN and also IPN-B are associated with the development of tubular adenocarcinoma, while MUC1-negativity usually underlies the development of colloid carcinoma during the progression of ICC. Aberrant expression of MUC1 is reported to relate to the invasive and metastatic properties of carcinoma cells[13,30], which is consistent with the notion that colloid carcinoma negative for MUC1 has a better prognosis than tubular adenocarcinoma with BilIN or IPN-B with increased expression of MUC1.

Expression of CKs

In most cases of IPN-B or BilIN, CK7 (biliary cytokeratin) is expressed regardless of the degree of atypia, and is present in more than 10% of the tumor cells. CK20 (intestinal cytokeratin) expression is infrequent in BilINs and ICC with BilIN, while it is frequent and extensive in the IPN-B series[10]. Taken together, CK7+/CK20- is common in BilINs and ICC with BilIN, whereas CK7+/CK20+ is common in IPN-Bs and ICC with IPN-B. IPN-B with colloid carcinoma frequently shows the CK7+/CK20+ pattern.

EXPRESSION PATTERNS OF CANCER-RELATED MOLECULES IN BILIN ANDIPN-B

G1-S modulators (p21, p53, cyclin D1, and Dpc4)

Phase G1-S of the cell cycle is disrupted in the development of malignant neoplasms which is characterized by uncontrolled cell proliferation[31,32]. As modulators of G1-S, p16, p21, p53, cyclin D1 and the retinoblastoma (Rb) gene product are all important[31]. Cyclin D1 forms a complex with cyclin-dependent kinase (CDK), which phosphorylates Rb. Phosphorylated Rb allows cells to enter the S phase[33]. P16 and p21, both of which are CDK

inhibitors, can prevent cell cycle progression at the G1-S checkpoint by binding to cyclin D1/CDK complexes[34]. P53 can up-regulate p21 expression under physiological conditions[35,36]. P53 is mutated in a large number of human malignant neoplasms, and mutated p53 is immunohistochemically detectable. Interestingly, these cell-cycle accelerants or decelerators are abnormally expressed not only in advanced malignant tumors, but also in non-invasive premalignant lesions. Transforming growth factor-β (TGF-β) has several biological functions, such as epithelial growth inhibition. TGF-β signaling is transmitted to the nucleus by cooperation with TGF-β receptors on cell membranes and intracytoplasmic mediators (Smads). Smad4/Dpc4 is one of the most important regulators involved in carcinogenesis. Inactivation of the DPC4 gene is frequently reported in some types of tumors, and a tumor-suppressive property is thus suggested[37,38].

In both the BilIN and IPNB series, the expression of p21, p53, and cyclin D1 is upregulated with histological progression, whereas the expression of Dpc4 is down-regulated[23,39,40]. In BilIN, p21 expression is significantly upregulated already at BilIN-1 and also at BilIN-2 and -3. In contrast, levels of all of these molecules change both gradually, and stepwise, in IPNB lineages. Interestingly, p53 expression patterns differ significantly between BilIN and IPNB. That is, p53 expression is dramatically upregulated at the invasive stage of BilIN, while it is quite low in noninvasive BilIN. In contrast, p53 expression is already upregulated in IPN-B1 and reaches a plateau in IPN-B2 and invasive ICC. Taken together, p21, p53, cyclin D1, and Dpc4 are involved in the carcinogenesis of both BilIN and IPN-B, though their expression patterns are not the same during the progression of IPN-B to ICC and BilIN to invasive ICC, suggesting different pathways in terms of cancer-related gene expression, too[9,10,18,23,39,40].

Polycomb group protein EZH2 and p16^{INK4a}

The *p16*^{INK4a} gene displays its tumor-suppressive function by binding and inactivating cyclin-dependent kinases. Therefore, multiple epigenetic and genetic mechanisms for silencing or overcoming these tumor-suppressive processes are required for the development and progression of malignant tumors. During the progression of IPN-B in hepatolithiasis, p16^{INK4a} expression is shown to be decreased stepwise with the progression through IPNB to invasive CC[23]. A methylation-specific polymerase chain reaction (MSP) showed that hypermethylation of the p16^{INK4a} promoter was signficantly involved in this decrease in the expression of p16^{INK4a}. Decreased expression of p16^{INK4a} was seen in cases of IPN-B1 with mild dysplasia, and continued with the progression of IPN-B2 to ICC. MSP revealed that about a half of IPN-B foci harbored a hypermethylated *p16*^{INK4a} promoter, and such foci were significantly correlated with decreased expression of p16^{INK4a} protein. Cell proliferative activities exhibited a stepwise increase from IPN-B1 to IPN-B2. Together, *p16*^{INK4a}'s inactivation, due mainly to its promoter hypermethylation, is a frequent and early event in cases of IPN-B, and may be related directly or indirectly to genetic and epigenetic alterations of other cell cycle regulators during the progression of IPN-B.

Recent studies showed that the Polycomb group proteins EZH2 and Bmi1 are chromatin-modifying enzymes, and interact with key elements that control cell growth and proliferation, such as the *p16*^{INK4a} gene, and that abnormal expression of EZHE and Bmi1 is involved in tumorigenesis, including malignant transformation. Aberrant expression of EZH2 is regarded as a potential marker of advanced or aggressive cancer with a poor prognosis, differing from benign or indolent counterparts. As for the participation of EZH2 in multi-step cholangiocarcinogenesis with respect to the tumor suppressor gene *p16*^{INKa}, it has been reported that the expression of p16^{INK4a}, which was frequent in BilIN1, was decreased in BilIN-2/3 and invasive carcinoma, suggesting that a certain mechanism represses p16^{INKa} expression with the progression of BilIN to invasive CC (Figures 4A and 5A)[24]. Interestingly, EZH2 expression showed a stepwise increase from BilIN-1, -2 and -3 to invasive carcinoma, suggesting that hypermethylation of the *p16*^{INK4a} promoter was related to the aberrant expression of EZH2 (Figures 4B and 5B)[24]. The knockdown of EZH2 in cultured CC cells decreased *p16*^{INK4a} methylation and decreased the binding of EZH2 to the *p16*^{INK4a} promoter, suggesting that direct binding of EZH2 is involved in the regulation of the *p16*^{INK4a} gene. It therefore seems conceivable that over-expression of EZH2 may induce hypermethylation of the *p16*^{INK4a} promoter, followed by decreased expression of p16^{INK4a} in the multi-step cholangiocarcinogenesis through the rhw BilIN lineage, particularly in cases of hepatolithiasis[22].

Matrix metalloproteinases (MMPs)

MMPs are believed to play a pivotal role in the malignant behavior of cancer cells such as rapid tumor growth, invasion, and metastasis, by degrading the extracellular matrix (ECM)[41]. MMP-7 is the smallest MMP expressed exclusively in the tumor cells themselves. MMP-7 has proteolytic activity against components of the ECM such as collagens, proteoglycans, laminin, and fibronectin[42]. MMP-7 overexpression was observed in malignant tumors, including carcinomas of the colorectum and stomach[43]. MT1-MMP is expressed on the cancer cell surface as an invasion-promoting proteinase[44]. One of its major targets is type I collagen. MT1-MMP also regulates cell-ECM interaction by processing cell adhesion molecules, and eventually promotes cell migration as well. Recently, we showed that MMP-7 and MT1-MMP were commonly expressed in invasive ICC with BilIN, but not non-invasive lesions (BilIN-1, -2, -3), suggesting that the expression of MMP-7 and MT1-MMP was closely associated with invasive growth of the BilIN lineage[25]. In contrast, the IPNB lineage was only occasionally and weakly positive for these molecules, reflecting its favorable prognosis[3,45,46].

Cell adhesion molecules (β-catenin and E-cadherin)

A reduction of β-catenin in the cell membrane reflects a disruption of cell-to-cell adhesion, and E-cadherin is one subtype of transmembrane glycoprotein whose cytoplas-

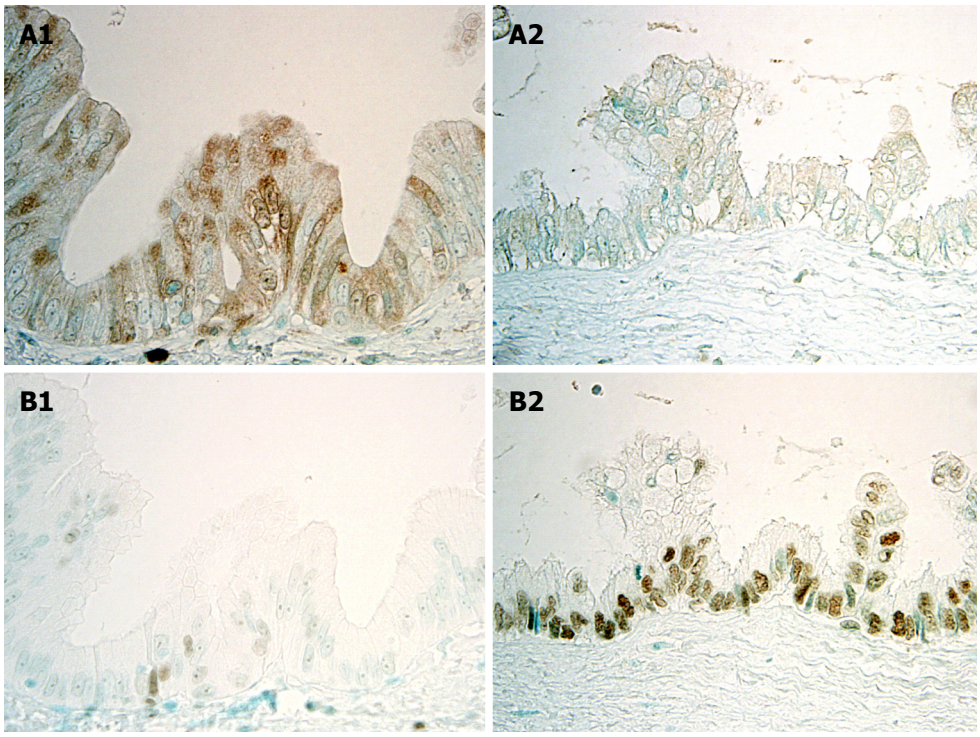

Figure 4 Immunohistochemical expression of p16^{INK4a} and EZH2 in BilIN-1 and BilIN-2. A: p16^{INK4a} is intensely and frequently expressed in the cytoplasm and nuclei in BilIN-1 (left), but such expression is unclear in BilIN-2 (right); B: While EZH2 is occasionally expressed in the nuclei in BilIN-1 (left), this expression increases markedly in BilIN-2 (right)[22].

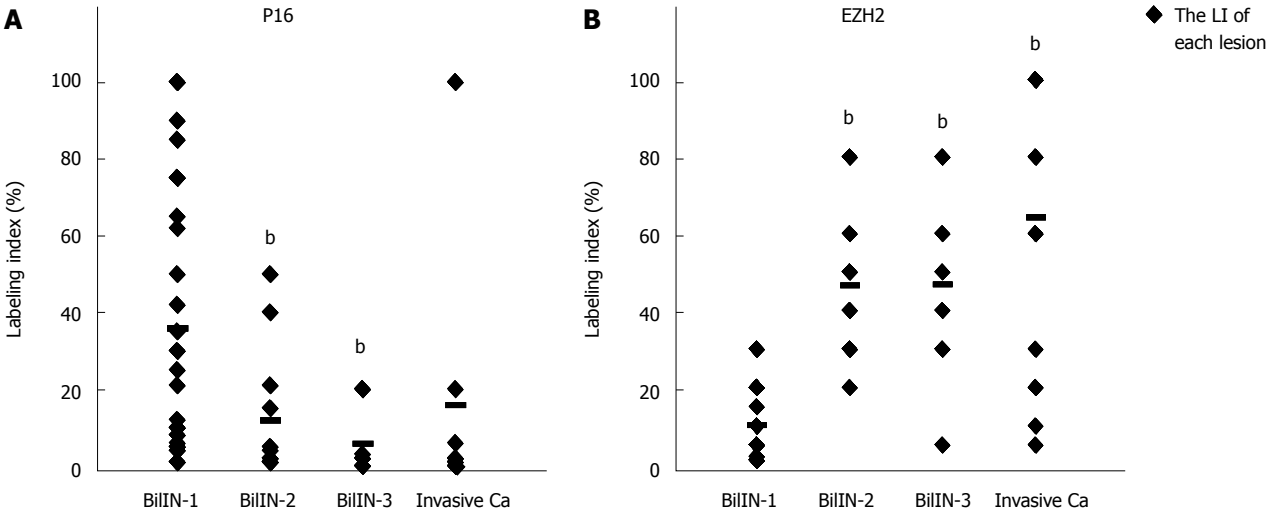

Figure 5 Semiquantitative evaluation of P16INK4a and EZH2 in BilIN-1, -2, and -3, and invasive cholangiocarcinoma (CC). The labeling index (LI) reflects the percentage of cells immunohistochemically positive for P16^{INK4a} or EZH2 in each lesion of BilIN-1, -2, and -3, and invasive CC. $^{b}P < 0.01$ *vs* BilIN-1. A: The LI of P16^{INK4a} expression (mean ± SD) decreases with the progressing of BilINs and is higher in invasive CC; B: Expression of EZH2 increases with the progression of BilINs and is greater in invasive CC[22].

mic domain is bound to α- and β-catenin. Interestingly, decreased membranous expression of β-catenin is correlated well with that of E-cadherin[25]. Decreased membranous expression of both these molecules is closely associated with the invasion of carcinoma cells, and is recognizable early on in both the BilIN and IPNB lineages. The membranous expression of β-catenin decreases with the progression of in both BilIN and IPN-B, particularly after the invasion and then metastasis of carcinoma cells. Membranous expression of β-catenin and E-cadherin decreased more markedly in ICC with BilIN than in ICC with IPNB, a finding compatible with the report of Sugimachi *et al*[47]. Interestingly, decreased membranous expression of E-cadherin and β-catenin in the BilIN and IPN-B lineages was associated with the aberrant expression of MMP-7 and MT1-MMP, suggesting that disruption of the membranous distribution of β-catenin and E-cadherin may result in conditions favorable for the invasion and metastasis of carcinoma cells of these two lineages which express MM-7 and MT1-MMP.

Cyclin D and c-myc

Cyclin D1 is known to bind to cyclin-dependent kinase (CDK) 2 or CDK 4, to act as a CDK inhibitor, and to inhibit Rb phosphorylation leading to an accelerated cell cycle progression and increased cell proliferation[48]. C-myc

has mitogenic effects modulating regulators of cell cycle progression[49]. Overexpression of cyclin D1 was reported in about a half of ICCs and was associated with poor histological differentiation and a poor prognosis, and overexpression of c-myc was reported in about a half of ICCs[50,51]. As for precursor lesions of ICC, increased expression of cyclin D1 and c-myc was more frequent in the IPNB lineage than in the BilIN lineage, and was possibly related to the Wnt signaling pathway associated with the nuclear accumulation of β-catenin. Cyclin D1 and c-myc, targets of Wnt signalling, were frequently positive in the IPNB lineage, and interestingly, nuclear β-catenin staining was observed only in the IPNB lineage. Decreased membranous expression of β-catenin and E-cadherin is an early event in the tumorigenesis of both the BilIN and IPNB lineages[52]. The Wnt signaling pathway may therefore play an important role in the tumorigenesis of the IPNB lineage.

CONCLUSION

In conclusion, two precursor lesions of invasive ICC arising in the intrahepatic large bile ducts, BilIN and IPN-B, were reviewed. Both types show a multistep progression followed by invasive carcinoma. Three carcinogenetic pathways from BilIN and IPN-B to ICC were proposed: BilIN progressing to tubular adenocarcinoma, and IPN-B progressing to tubular adenocarcinoma or to colloid carcinoma. These three pathways progress through characteristic multistep genetic and epigenetic processes.

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GUIDELINES FOR CLINICAL PRACTICE

Combination treatment in HBeAg-negative chronic hepatitis B

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Abstract

Chronic hepatitis B (CHB) represents an important public health problem. HBeAg-negative CHB is frequently associated with advanced liver disease and its prevalence is increasing. Monotherapy with either interferon (conventional or pegylated) or nucleoside/nucleotide analogues has its limitations. It has been suggested that a combination of these agents might increase antiviral efficacy. However, existing data do not support this hypothesis, even though combination treatment appears to reduce the risk for emergence of lamivudine resistance. Nevertheless, most existing combination studies are small, and it is possible that they have not been designed to detect significant differences between combination treatment and monotherapies. Another limitation of these studies is that, in most of them, lamivudine treatment was discontinued after 1 year, a strategy that is not followed in clinical practice. It was thought to be interesting to evaluate the combination of a short course of interferon (particularly pegylated) with the long-term administration of nucleotide or nucleoside analogues. The efficacy of combining pegylated interferon with the newer nucleotide or nucleoside analogues or of nucleotide with nucleoside analogues could also be evaluated. However, findings show that until more data are available, combination therapy cannot be recommended as first-line treatment in patients with CHB. On the other hand, add-on therapy with adefovir or tenofovir is the treatment of choice in patients who develop resistance to lamivudine. In patients with cirrhosis, a combination of lamivudine/adefovir may also be used as initial treatment; another option would be to add tenofovir in patients with an insufficient response to entecavir.

Key words: Interferon; Lamivudine; Adefovir; Telbivudine; Entecavir; Tenofovir; Combination; Monotherapy; HBeAg negative chronic hepatitis B; Resistance

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INTRODUCTION

Chronic hepatitis B (CHB) represents an important public health problem[1]. It is estimated that 350 million people worldwide are affected by CHB, and 500 000 to 1.2 million patients die each year of the complications (mainly cirrhosis and hepatocellular carcinoma) of CHB[1]. HBeAg-negative CHB is characterized by the presence of the hepatitis B virus (HBV) with mutations in the precore or basic core promoter region, which prevent the synthesis of HBeAg[2]. The prevalence of HBeAg-negative CHB appears to be increasing; median rates are 33% in Europe (range: 10-72), 14% in the United States (range: 13-22) and 15% in Asia (range: 5-47)[3]. Patients with HBeAg-negative CHB are more likely to have severe liver disease at the time of diagnosis, and to progress to cirrhosis than patients with

HBeAg-positive CHB[2]. The management of HBeAg-negative CHB is therefore an important aspect of the treatment of CHB.

LIMITATIONS OF MONOTHERAPY IN CHB

Seven agents are currently approved for the treatment of CHB, including interferon [conventional interferon (IFN) and pegylated (PEG) IFN α-2a] and the nucleoside/nucleotide analogues [lamivudine (LAM), adefovir (ADV), telbivudine, entecavir and tenofovir][4,5]. IFN stimulates cell-mediated immune responses against HBV, whereas the nucleoside/nucleotide analogues inhibit HBV replication by acting on the HBV polymerase[6]. The advantage of IFN is that it is administered for a defined period (16-48 wk), induces sustained responses in some patients and is not associated with the development of HBV resistance[4,5]. However, IFN treatment requires subcutaneous injections and is frequent associated with side effects[4,5]. The nucleoside/nucleotide analogues are given orally and are well tolerated[4,5]. However, stopping these agents leads to a rebound of HBV replication in most cases and they therefore have to be given for long periods of time and probably indefinitely[4,5].Unfortunately this strategy is compromised by the development of HBV resistance[4,5].

CONVENTIONAL IFN PLUS LAM COMBINATION

It is clear that monotherapy with either IFN or nucleoside/nucleotide analogues have limitations. Since these agents have different mechanisms of action, it has been suggested that combining them might result in more potent and sustained suppression of viral replication[7]. This might in turn also reduce the risk for development of antiviral resistance[7]. Accordingly, several studies compared IFN/LAM combination with LAM monotherapy in patients with HBeAg-negative CHB[8-12]. End-of-treatment virological and biochemical response rates with the 2 treatments were either similar[9,12] or higher with IFN/LAM combination[8,11]. However, sustained (i.e. after treatment discontinuation) virological and biochemical response rates were similar with IFN/LAM combination and LAM monotherapy in all studies[8,10-12]. Moreover, improvement in liver histology was either similar in the 2 groups[12] or greater with LAM monotherapy[9,10]. Development of LAM-resistant mutants was found to be more frequent in patients treated with LAM monotherapy in some[8,11] but not all studies[9,10,12]. Treatment discontinuation rates were similar in the 2 groups[8,11,12]. Interestingly, the administration of higher doses of IFN (up to 10 MU tiw), or for longer periods (up to 24 mo) was not associated with better results of IFN/LAM combination[8-12].

PEG-IFN PLUS LAM COMBINATION

Pegylated IFN appears to be more effective than conventional IFN and has a more convenient dosing scheme (one weekly administration *vs* tiw with conventional IFN)[5]. A limited number of studies have evaluated the effectiveness of a PEG-IFN/LAM combination compared with LAM monotherapy in patients with HBeAg-negative CHB[13-15]. In the largest study, Marcellin *et al*[13] randomly assigned 537 patients to 48 wk of treatment with a PEG-IFN α-2a/LAM combination, PEG-IFN α-2a monotherapy or LAM monotherapy. After a follow-up time of 24 wk after discontinuing treatment,those patients treated with the PEG-IFN α-2a/LAM combination showed higher virological and biochemical response rates than those treated with LAM monotherapy[13]. However, response rates were similar in the PEG-IFN α-2a/LAM combination and PEG-IFN α-2a monotherapy patient groups[13]. Moreover, rates of histological response were similar in the 3 groups[13]. The rates of adverse events were similar in the PEG-IFN α-2a/LAM combination and PEG-IFN α-2a monotherapy groups but significantly lower in the LAM monotherapy group[13]. Treatment with PEG-IFN α-2a/LAM combination was associated with lower rates of emergence of LAM resistance compared with LAM monotherapy[13]. In 2 smaller studies (n = 48 and n = 126, respectively PEG-IFN α-2b/LAM combination and PEG-IFN α-2b monotherapy administered for 48 wk resulted in similar end-of-treatment and sustained biochemical and virological response rates 24 wk later[14,15]. Liver biopsy was not performed nor were rates of emergence of LAM resistance reported in the latter studies[14,15].

STAGGERED IFN/LAM COMBINATION

In vitro studies have suggested that LAM restores HBV-specific cytotoxic T lymphocyte reactivity[16,17]. It was therefore hypothesized that LAM pre-treatment might increase responsiveness to subsequent IFN administration[16,17]. Accordingly, a number of studies have evaluated a staggered treatment scheme, with 1-6 mo of LAM monotherapy preceding treatment with 1-12 mo of a IFN/LAM combination[12,18-21]. In some studies, the IFN/LAM combination was followed by 6 mo of LAM monotherapy[19] or 6 mo of IFN monotherapy[18,20]. The IFN/LAM combination was compared with LAM monotherapy[12,21], IFN monotherapy[18] or both[19]. End-of-treatment virological and biochemical response rates were similar in the IFN/LAM combination and LAM monotherapy groups[12,19-21] and higher than those of the IFN monotherapy groups[18,19]. In most studies, after 6-27 mo of follow-up, sustained virological and biochemical response rates were also similar in the IFN/LAM combination and the LAM monotherapy[12,19,21] or IFN monotherapy groups[18,19]. The biochemical response rates were higher in the IFN/LAM combination group at 24 wk after treatment discontinuation in only in one study, but virological response rates did not differ

significantly between the 2 groups[20]. LAM resistance was observed more frequently in the LAM monotherapy group in most[19-21] but not all studies[12]. Improvement in liver histology was similar with the IFN/LAM combination and LAM monotherapy[12,19,21] but less with IFN monotherapy[19]. Treatment discontinuation rates were similar with the IFN/LAM combination and LAM monotherapy[19-21]. Only 1 study evaluated a staggered combination scheme including PEG-IFN in patients with HBeAg-negative CHB[22]. In this report, 18 patients were treated with a LAM/PEG-IFN α-2b combination (3 mo LAM, 3 mo LAM/PEG-IFN α-2b and 9 mo PEG-IFN α-2b alone) and 24 patients were treated with LAM alone for a median of 25 mo[22]. At the end of the treatment, virological and biochemical response rates did not differ significantly between groups[22]. At 12 mo after treatment discontinuation, virological response rates did not differ significantly between the 2 groups but biochemical response rates were higher in the LAM/PEG-IFN α-2b combination group[22]. LAM resistance was observed more frequently in the LAM monotherapy group[22]. Finally, only 1 study compared a staggered combination scheme (12 wk of LAM monotherapy followed by 40 wk of an IFN/LAM combination) with a IFN/LAM combination for 52 wk[12]. This study also included a group treated with LAM monotherapy for 52 wk[12]. End-of-treatment and end-of-follow-up virological and biochemical response rates, changes in liver histology and rates of development of LAM-resistant mutants were similar in the 3 groups[12].

SEROCONVERSION TO ANTI-HBS WITH COMBINATION TREATMENT

Loss of HBsAg and seroconversion to antiHBs indicates resolution of CHB and is considered as a complete response to treatment[23]. However, this result is rarely achieved with monotherapy with either IFN or nucleoside/nucleotide analogues[23]. Some uncontrolled studies suggested that IFN combined with either LAM or ADV is associated with high rates of seroconversion to antiHBs[24,25]. However, in the controlled studies there were no differences in rates of HBsAg loss or seroconversion to antiHBs between IFN (conventional or PEG-IFN) and LAM combination (either synchronous or sequential) and either monotherapy[8-15,18-22].

COMBINATION TREATMENT WITH DIFFERENT NUCLEOSIDE AND NUCLEOTIDE ANALOGUES

Combination treatment with a nucleotide analogue (lamivudine or telbivudine) and a nucleoside analogue (e.g. adefovir or tenofovir) might be a therapeutic option because these agents have different resistance profiles[26]. A limited number of studies have assessed the effects of combining nucleotide and nucleoside analogues in patients with HBeAg-positive CHB[27-29]. The use of ADV, combined with either LAM or emtricitabine (which is not yet approved in CHB) was shown to reduce HBV DNA levels more than ADV monotherapy[27,28]. In contrast, combining telbivudine and LAM (i.e. 2 nucleotide analogues) was not shown to be more effective than telbivudine monotherapy[29]. In the only study which evaluated the combination of oral antiviral agents in patients with HBeAg-negative CHB, 163 patients with CHB (48% HBeAg-negative) were treated with a combination of emtricitabine plus clevudine or emtricitabine monotherapy[30]. Neither of these agents is yet approved in CHB. At the end of the 24-wk treatment period, virological and biochemical response rates were similar in the 2 groups[30]. However, 24 wk after treatment discontinuation, virological and biochemical response rates were higher in the emtricitabine/clevudine combination group[30]. Emtricitabine-resistant mutants emerged at the same rate in the 2 groups[30]. Adverse events were similar in the 2 groups but post-treatment exacerbation of CHB occurred less frequently in the emtricitabine/clevudine combination group[30]. In a study recently presented in abstract form, a tenofovir/emtricitabine combination was shown not to be more effective than tenofovir monotherapy in patients with persistent viral replication, despite treatment with ADV (n = 105, 27% HBeAg-negative)[31].

CONCLUSION

In conclusion, available data do not support the hypothesis that combination treatment improves virological or biochemical response rates compared with monotherapy in HBeAg-negative CHB. Combination treatment might also reduce compliance and increase the cost of therapy, as well as the risk of incurring side effects and drug interactions. On the other hand, the advantage of combination treatment is that it appears to reduce the risk for development of LAM resistance. However, most studies are small and it is possible that they were not designed to detect significant differences between a IFN/LAM combination and LAM monotherapy. In addition, the inclusion of non-responders to previous IFN treatment might have also reduced the efficacy of the IFN/LAM combination. Another limitation of these studies is that LAM treatment was discontinued after 1 year in most of them, a strategy that is not normally followed in clinical practice. It would be interesting to evaluate the combination of a short course of IFN (particularly PEG-IFN) with long-term administration of nucleotide or nucleoside analogues. The efficacy of combining PEG-IFN with the newer nucleotide or nucleoside analogues or of nucleotide with nucleoside analogues should also be evaluated. Until more data are available, combination therapy cannot be recommended as first-line treatment in patients with CHB. However, combination therapy is the treatment of choice in patients who develop resist-

ance to LAM, where tenofovir or ADV should be added to LAM[5]. Moreover, in high-risk patients (particularly in those with cirrhosis), it might be beneficial to add tenofovir in patients with an insufficient response to entecavir. Finally, in patients with decompensated cirrhosis, a LAM/ADV combination could be used as initial treatment, to achieve rapid suppression of HBV replication and to reduce the risk of resistance[5].

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REVIEW

Surveillance and diagnosis of hepatocellular carcinoma in patients with cirrhosis

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Abstract

Early identification of hepatocellular carcinoma (HCC) is more frequent because of surveillance programs for HCC worldwide. The optimal strategy of surveillance in cirrhosis is a current topical issue. In terms of diagnosis, recent advances in non-invasive imaging technology, including various techniques of harmonic ultrasound, new ultrasound contrast agents, multi-slice helical computed tomography and rapid high quality magnetic resonance, have all improved the accuracy of diagnosis. Consequently the role of liver biopsy in diagnosis of HCC has declined. The imaging diagnosis relies on the hallmark of arterial hypervascularity with portal venous washout. However, with recent advances in genomics and proteomics a great number of potential serum and tissue markers have been identified and are being developed as new candidate markers for both diagnosis and prognosis of hepatocellular carcinoma, and may increase the need for liver biopsy.

Key words: Hepatocellular carcinoma; Liver neoplasm; Screening; Diagnosis; Imaging; Liver biopsy; Molecular markers

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy worldwide and it represents the leading cause of death in patients with cirrhosis in Europe[1]. Malignant hepatic cell transformation is more frequent in cirrhotic livers, accounting for 80%-90% of overall autopsied series[2]. Between 59% and 94% of new diagnosed nodules in cirrhosis are histologically characterized as malignant[3,4] and about 50% of the hemangioma-like lesions in cirrhosis are shown to be HCC[5]. It is reasonable and common clinical practice to consider any lesions in a cirrhotic liver, as malignant until proven otherwise.

In developed countries there are surveillance programs for "at-risk" people, including those with cirrhosis, to identify the malignant lesions when they are small. For this purpose differentiating between early HCC and a dysplastic nodule is an important issue in a routine clinical setting.

Recent advances in non invasive imaging technology for the diagnosis of hepatocellular carcinoma include various techniques of tissue harmonic ultrasound (US) imaging, new US contrast agents, multi-slice helical computed tomography (CT) and rapid high quality

magnetic resonance (MRI) with new, tissue-specific contrast agents.

Ultrasonography is the first line of investigation in the detection of focal liver lesions, particularly as it used for surveillance of HCC in patients with cirrhosis, as it has relatively low cost, is non invasive and has wide spread availability[6].

SURVEILLANCE AND SCREENING

Surveillance of HCC in patients with cirrhosis in most centres is performed using 6-monthly US and in some centres this is combined with α-fetoprotein (AFP).

Data to support the effectiveness of ultrasound surveillance are sparse because of ethical problems of not performing ultrasound as it is part of current clinical practise[7-10].

The value of using AFP for surveillance has not been validated but once a nodule has been detected is useful[11]. The AFP test, with a cut-off value of 20 ng/mL, has a sensitivity from 41% to 65%[11-16]: lowering the cut-off value and changing it for different etiologies of liver disease, such as in HBV carriers results in greatest sensitivity[17]. Currently, HCC screening with AFP alone is not recommended, except when US either is not available or of poor quality[18]. Moreover AFP measurement together with US screening, is not cost-effective[19], as it only increases sensitivity by about 10% compared to US screening alone[20].

However, high levels of AFP can identify an "at risk" category of patients with cirrhosis that require surveillance[21] (Table 1).

In the sole randomized controlled trial performed in China, which also included individuals without cirrhosis, the survival rate at five years after enrolment was 46.4% in the surveillance group (AFP plus US scan every 6 mo) against 0% in the control group[22].

Indirect proof of the utility of a surveillance strategy is the resulting change in presentation of HCC, with an increased rate of detection of tumors < 2 cm in diameter. In fact while tumors less then 2 cm in diameter represented less than 5% of the cases in the early nineties in Europe, now they represent up to at least 30% in Japan[23]. However, the increased diagnosis of HCC does not necessarily mean an improvement in survival[24], although a well documented cohort study does suggest this[10]. More data are need to substantiate the value of this strategy. Based on the estimated HCC doubling time and cost-effectiveness estimates, the recommended screening interval is 6 mo, although a 1 year interval seems to be as effective[25].

An additional consideration is the fact that ultrasound imaging requires good equipment and skilled operators. In a retrospective study in patients with cirrhosis five-year survival was better in the group screened in a specialized centre (52%) *versus* the group screened in non-specialized centres (40%)[26]. Surveillance programs for HCC would benefit from the same organizational setup as breast programs, with recall facilities and dedicated centres.

Table 1 At risk population for HCC surveillance: AASLD guide lines[21]

Hepatitis B carriers
Asian males 40 years or more
Asian females 50 years or more
All cirrhotic hepatitis B carriers
Family history of HCC
Africans over age 20
For non-cirrhotic hepatitis B carriers not listed above the risk of HCC varies depending on the severity of the underlying liver disease, and current and past hepatic inflammatory activity. Patients with high HBV DNA concentrations and those with ongoing hepatic inflammatory activity remain at risk for HCC
Non-hepatitis B cirrhosis
Hepatitis C
Alcoholic cirrhosis
Genetic hemochromatosis
Primary biliary cirrhosis
Group with lack of evidence. Although the following groups have an increased risk of HCC no recommendations for or against surveillance can be made because a lack of data precludes an assessment of whether surveillance would be beneficial: α1-antitrypsin deficiency, non-alcoholic steatohepatitis, autoimmune hepatitis

Ultrasound surveillance as it is currently practised has an acceptable sensitivity of 65%-80% and has a upper level of specificity of more than 90%[7,21,27]. Tumor size significantly affects the sensitivity of US in detecting HCC. Sensitivity ranges from 42% for lesions smaller than 1 cm[28,29] to 95% for tumors of larger size[30]. In pre-transplant screening, the US sensitivity is poor because of the coarse echotexture of the liver, the frequent presence of ascites and the high rate of malignant lesions present in end-stage liver disease[31]. In a retrospective study on 200 patients who underwent liver transplantation, within 3 mo of previous screening, the US scanning was correlated with explanted livers, and had a sensitivity of only 13.6% to 50% for lesions of 1-5 cm in diameter[32]. Therefore in liver transplant candidates, CT or MRI scanning should be performed[33].

Using grey-scale US more than 76% of hepatocellular cancers smaller than 2 cm appear as hypoechoic, with or without posterior enhancement[34]. About 17% of small HCC show an hyperechoic appearance[34,35], features related to the fatty changes occurring during the evolution of the borderline lesion. Fewer small HCC lesions appear isoechoic[34,35]. Lesions larger than 2 cm in diameter show a more heterogeneous pattern than smaller lesions because of the changes during the growth of the lesion (i.e. development of necrotic hypoechoic areas, calcifications and pseudocapsule). In these cases the presence of the "halo sign" and posterior enhancement increase the specificity of diagnosis[21,27,31].

The use of doppler US or power doppler US may help establish the nature of the lesion by detecting

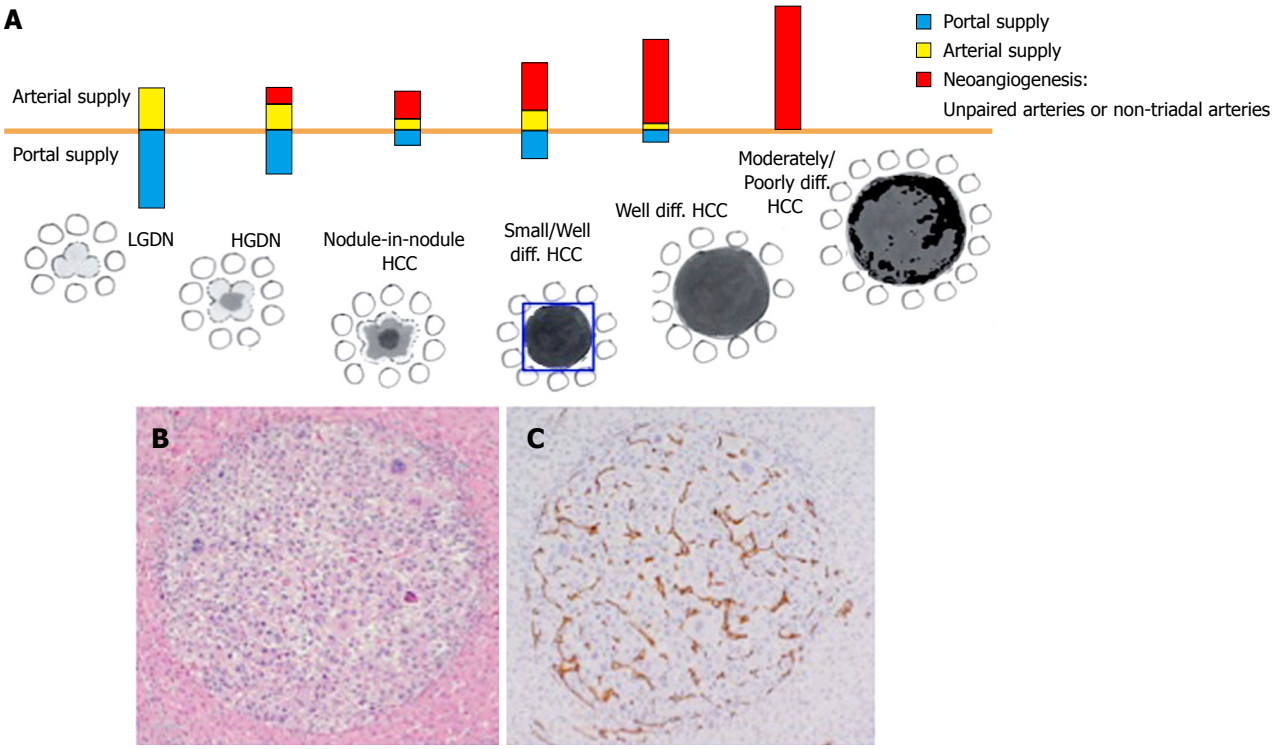

Figure 1 The diagram shows the changes of intranodular blood supply that characterises HCC (A); The sampled small/well differentiated HCC shows in HE (B) compact carcinomatous tissue well circumscribed from dysplastic tissue; CD34 immunostain of the same nodule (C) demonstrates arteries not confined to portal tracts in HCC. diff.: Differentiated.

arterial vascularization. However, in small HCC and in lesions located deep within the liver parenchyma, the sensitivity of doppler is low and a typical arterial pulsatile flow arterial pattern is detected in only 50% of nodules [31,36]. Colour or power Doppler US of large HCC often demonstrate a basket pattern, with a fine blood network surrounding the nodule and flowing into it[37].

When new nodules are found in a cirrhotic liver, these must be characterized with a contrast-enhanced imaging technique. During surveillance using double harmonic-equipped US machine, the same operator can also diagnose an HCC with contrast-enhanced US (CEUS). This make CEUS more cost-effective[38]. However, MRI or CT are needed whenever a second technique of imaging is needed, particularly if the lesions is 1-2 cm in diameter, and to image the whole liver to ensure there are no other lesions. A chest CT can be done at the same time, to exclude the presence of metastases.

DIAGNOSIS-IMAGING BASED ON BLOOD SUPPLY AND TISSUE CHARACTERIZZATION

In 2001 the European Association for the Study of the Liver (EASL) published a consensus statement defining histological and radiological criteria for the identification of HCC, and categorizing patients with cirrhosis and HCC on the basis of the size of presumed nodule[18]. In 2005, practice guidelines deriving from EASL, American Association of Study for the Liver Diseases (AASLD) and Japanese Society of Hepatology (JSH) revised the 2001 statements[21], giving prime diagnostic importance to arterial hypervascularization together with portal venous washout, and adding CEUS as a non invasive diagnostic technique[21,39].

In the multistep process of hepatocarcinogenesis there is a progressive change in the vascular supply that consists of an increase in arterial blood supply and loss of portal blood. Contrast enhancing agents can characterize the vascular pattern of focal liver lesions secondary to hepatocellular transformation with a good correlation between grade of HCC malignancy and intranodular hemodynamics[40,41] (Figure 1).

The typical feature of HCC, demonstrated by using intravenous contrast media that show extracellular-vascular distribution, consists of arterial enhancement with early portal and venous phase wash-out. Imaging techniques recommended in EASL/AASLD/JSH guidelines[21] are: triphasic CT with iodinated contrast media (but not lipiodol because of its low sensitivity), dynamic MRI enhanced by gadolinium or manganese based media (that have also a slightly hepatocellular uptake with biliary excretion) and CEUS with microbubble contrast agents[21]. Conventional angiography and CT hepatic arteriography-portography can be used, but are rarely necessary for diagnosis.

The average sensitivity, specificity and especially the predictive positive values of these techniques are currently comparable (Table 2).

Table 2 Overall sensitivity, specificity and predictive value of imaging technique for the diagnosis of hepatocellular carcinoma

Study	Nunber of patients (*n*)	HCC patients/ HCC instances	Gold standard reference	Imaging technique	Sensitivity (%)	Specificity (%)	PPV (%)	Ref.
Ward *et al* (2000)	145	25/76	Explant/ biopsy	MR (SPIO)	66	NA	NA	[72]
				MR (Double)	80	NA	93.5	
Rode *et al* (2001)	43	18/13	Explant	SDCT	53.8	92.9	94.3	[58]
				MR (Gd)	76.9	57.1	42.3	
Krinsky *et al* (2001)	71	10/19	Explant	MR (Gd)	53	NA	96.9	[69]
Krinsky *et al* (2002)	24	24/> 118	Explant	MR (Gd)	33	NA	NA	[70]
De Ledingen *et al* (2002)	34	21/54	Explant	MDCT	51.9	84.6 pts[2]	89.5	[55]
				MR (Gd)	61.1	100[2]	100	
Libbrecht *et al* (2002)	49	17/77	Explant	MDCT	70 pts[1]	82[2]	NA	[57]
				MR (Gd)	50[1]	79[2]	NA	
Zacherl *et al* (2002)	23	23/50	Explant	MDCT	75	NA	64	[62]
Barthia *et al* (2003)	31	14/32	Explant	MR (Double)	78	NA	NA	[31]
Burrell *et al* (2003)	50	29/76	Explant	MDCT	61	NA	87	[3]
				MR (MRA)	76	NA	90	
Teefey *et al* (2003)[3]	22	9/18	Explant	MDCT	57-67 pts[1]	69-75[2]	NA	[60]
				MR (Gd)	50-56[1]	63-81[2]	NA	
Battakiarjya *et al* (2004)	30	30/46	Explant	MDCT	67.4	78.9	NA	[53]
				IOCT	68	88.6	NA	
Kim *et al* (2004)	27	27/50	Biopsy/clinical/ radiological	MR (Gd)	91.3	NA	92.6	[68]
				MR (SPIO)	77.3	NA	NA	
Valls *et al* (2004)	85	51/85	Explant	MDCT	78.8 pts	NA	88	[61]
Kim *et al* (2006)	46	31/53	Biopsy/clinical/ radiological	MDCT	77.4-79.2	NA	95-97	[56]
				MR (Gd)	92.5-94.3	NA	92-96	
Hecht *et al* (2006)	38	18/19	Explant	MR (Gd)	68.4	65.7	NA	[67]
Ronzoni *et al* (2007)	88	48/139	Explant	MDCT	64	NA	66.9	[59]
					73.3	NA	79	
Lauenstein *et al* (2007)	115	27/36	Explant	MR (Gd)	77.8	NA	NA	[71]
Forner *et al* (2008)	89	60/60	Biopsy	MR (Gd)	61.7	96.6	NA	[42]
				CEUS	51.7	93.1	NA	
Dai *et al* (2008)	498	NA/56	Biopsy/ resection	MDCT	80.4	97.9	NA	[52]
				CEUS	91.1	87.2	NA	
Choi *et al* (2008)		47/41	Explant	MDCT	65	NA	NA	[54]
				MR (Gd)	83	NA	NA	

[1]Patient-based sensitivity; [2]Patient-based specificity; [3]Two observers; [4]Three observers. CEUS: Contrast-enhanced ultrasonography; Gd: Gadolinium; HCC Hepatocellular carcinoma; IOCT: Ionized oil computed tomography; MDCT: Multi-detector computed tomography; MR: Magnetic resonance; MRA: Breath-old 3D gadolinium-enhanced angiography; MR (Double): Double-contrast MR with gadolinium and superparamagnetic iron oxide agents; NA: Not applicable; PPV: Positive predictive value; pts: patients; SDCT: Single-detector computed tomography; SPIO: Superparamagnetic iron oxide.

Part of the current recommendations[21], is that the diagnosis of hepatocellular carcinoma when the focal liver lesion is larger than 2 cm in diameter can be confidently made using one dynamic imaging technique which demonstrate the typical pattern of HCC (Table 3). Moreover if AFP serum level is > 200 ng/mL and the radiological appearance of the lesion is suggestive of HCC, the likelihood that the lesion is HCC is high, even without classical vascular enhancement and washout[21].

When there are nodules between 1 and 2 cm in diameter two concordant dynamic imaging techniques are needed to confirm HCC[21] (Table 3).

Only one prospective study has been published validating the international guidelines for nodules smaller than 20 mm in diameter[42]. In this study, MRI and CEUS were concordant for HCC in only 33.3% of cases, showing a poor predictive negative value of 42% (slightly higher if only considering lesions of more than 1 cm in diameter). In addition, commenting on the above-quoted study, Caturelli *et al*[43] suggest that the first diagnostic approach to a newly found liver lesion smaller than 20 mm in diameter should be a fine-needle aspiration, as this technique reaches a higher diagnostic accuracy than that reported for the two concordant imaging techniques[42,43]. Otherwise these lesions should not be treated as HCC, without histological evidence, as the false positive rate is about 20%[44]. Thus a biopsy of the lesion is required if confirmation of diagnosis of HCC is needed. Alternatively repeat imaging in 2-3 mo may resolve the issue.

Table 3 Newly found focal liver lesion in patients with cirrhosis. Screening and diagnosis: AASLD guide lines[21]

Focal lesion < 1 cm diameter: screen every 3-4 mo
Focal lesion 1-2 cm diameter: HCC diagnosed when 2 dynamic imaging techniques are concordant for HCC feature
Focal lesion > 2 cm diameter: HCC diagnosed with feature of HCC on 1 dynamic imaging technique

Lesions < 1 cm in diameter may be especially difficult

to characterize, even with the best imaging techniques. These small nodules are less likely to be HCC, even if they show hypervascularity with imaging techniques. These nodules need to be followed-up with US every 3-4 mo in order to determine if there is growth suggestive of malignant transformation (Table 3). If the nodule enlarges during follow-up, the criteria related to the particular size reached, pertain[21]. For this size of lesion, histology may not able to confirm the diagnosis.

Regenerative nodules and borderline lesions, such as dysplastic nodules and early HCC, show an inconsistent pattern of vascular enhancement. Benign regenerative nodules can also be hypervascular and if their diameter is between 1 and 2 cm, they should biopsied to resolve the diagnosis.

In addition, in smaller lesions the amount of Kupffer cells and fatty changes can be very variable, in comparison to overt HCC[45]. MRI with the use of super paramagnetic iron oxide (SPIO) enhancement, can be useful to characterize these lesions.

Characterisation of these smaller nodules poses a diagnostic challenge as they are more difficult to characterize even with pathological examination[23], although stromal invasion by "carcinomatous" cells, is associated with malignancy.

DIAGNOSIS - CEUS, CT AND MRI AS SINGLE TECHNIQUES: ADVANTAGES AND LIMITS

CEUS

In ultrasonography the main advance has been contrast-enhanced ultrasonography, which has improved the accuracy of ultrasound in detecting focal lesions, combining morphological aspects with functional perfusion ones[46]. CEUS is also very useful for assessment of HCC after treatment and has a good correlation with CT findings[47]. After trans-arterial embolization using lipiodol-based compounds that leave a radio-opaque shadow, only CEUS or dynamic MRI can detect residual vascularity.

Several reports have shown that CEUS is a good tool to show arterial hypervascularity of HCC. Two studies showed that CEUS has a higher detection rate compared to CT for lesions ⩽ 2 cm (53.6% *vs* 42.9% and 61% *vs* 49%)[48,49]. However, a more recent study, comparing CEUS with MRI, found that CEUS was slightly inferior to dynamic MR imaging in showing the presence of arterial hypervascularity (78% *vs* 85%)[42]. The sole detection of arterial hypervascularity without contrast wash-out in a small solitary lesion of ⩽ 2 cm in the setting of cirrhosis has a specificity of 86.2% and a positive predictive value of 92.2% for the diagnosis of HCC and thus cannot be considered as a conclusive finding[42]. Therefore, to increase the specificity of diagnosis it is necessary to evaluate contrast wash-out during the portal venous and the late phase as is currently recommended. Wang *et al*[50] found that the combination of arterial phase enhancement and the contrast wash-out during the portal venous and the late phase determined by CEUS are more specific for HCC if nodules < 2 cm, compared with the use of either arterial phase enhancement or the absence of delayed phase enhancement considered separately (91.7% *vs* 66.7%).

Considering CT as the gold standard, the sensitivity of CEUS in detecting HCC decreases with smaller tumoral lesions. Two studies have found that lesion base sensitivity was 89.3% for nodules < 2 cm and 67% for nodules < 1 cm[51]. In a recent study, 72 patients with cirrhosis with 103 small hepatic nodules (1-2 cm) detected on US, underwent CEUS and CT. Nodules which had contrast enhancement during the arterial phase and contrast wash-out during the late phase on CEUS or CT were diagnosed as HCC. According on these diagnostic criteria the sensitivity of CEUS was 91.1% and specificity 87.2%[52].

CT

Helical CT and more recently multi-detector helical CT (MDCT), which has improved spatial and temporal resolution, has increased the accuracy of CT in diagnosis of HCC[47]. Several studies, most using correlation with explanted liver after transplantation, show that the overall sensitivity, specificity and positive predictive value of CT in diagnosis of HCC ranges from 51.9%-80.4%, 78.9%-97.9% and 88.6%-92.9% respectively[52-62].

Among studies that have assessed the sensitivity of CT in diagnosis of HCC and stratified for tumor size, the sensitivity for HCC < 2 cm in diameter was 61%, for HCC of 1-2 cm it ranged from 53.3% to 76%[3,53,58,59,61], and for HCC <1 cm in diameter it ranged from 10% to 57%[3,53-56,58,59].

CT requires intravenous iodinated contrast material and exposes patients to ionizing radiation. Although induction of renal failure by contrast is low, patients with liver cirrhosis frequently have renal dysfunction limiting its use[63], and the efficacy of N-acetylcysteine for preventing contrast induced-nephrotoxicity is not substantiated[64].

MRI

The application of MRI for liver imaging continues to expand with the recent progress of rapid, high-quality scanning techniques and the development of new tissue-specific contrast agents. Although initially used to complement CT in selective applications, MRI now plays an important primary role (after US) for the detection and characterization of liver tumors[65]. Extracellular intravenous contrast agents and novel tissue specific contrast agents are used to assess patterns of enhancement. Mostly, gadolinium-chelates are used for MRI, but hepatocyte-targeted and reticolo-endothelial system-targeted compounds are also used. Many studies have been published on the sensitivity and specificity of MRI imaging for diagnosis of HCC[3,42,54,55,57,58,60,66-72]. Among studies with liver explant correlation, the sensitivity, specificity and positive predictive value ranged from 33%

to 83%, from 57.1% to 100% and from 42.3% to 100% respectively[3,54,55,57,58,60,66,67,69-72]. Studies which evaluated the sensitivity of MRI in detecting HCC, using only liver biopsy or clinical and radiological findings as gold standard, have shown an overall sensitivity, specificity and positive predictive value of 77.3% to 94.3%, 96.6% and of 92.6% to 97% respectively[42,56,68].

For MRI, the lesion-based sensitivity stratified for tumor size was 55.6% for < 2 cm in diameter; for HCC 1-2 cm in diameter it ranged from 52% to 89%[3,58,69,70], and for HCC <1cm in diameter it ranged from 4% to 88.2%[3,54-56,58,66,69,70,72].Super paramagnetic iron oxide particles used alone[56] or combined with gadolinium-based contrast agents[66,2] are highly sensitive for the diagnosis of small HCC. Ward *et al*[72] found a sensitivity of 91% for HCC ⩾ 1 cm and of 46% for HCC ⩽ 1 cm with double-contrast MRI. Another study [66] showed a sensitivity of 92% for HCC between 1 and 2 cm and 38% for HCC ⩽ 1 cm. The sensitivity of MRA was shown to be superior to CT, but it also decreased with size of tumor (84% for nodules between 1 and 2 cm; 32% for nodules < 1 cm) [3]. In contrast when gadolinium-enhanced multiphase dynamic MRI was compared with MDCT scanning, for detecting small HCC, the sensitivity of detection for HCC <1 cm was higher with MDCT than with MRI (90%-95% *vs* 70%-85%)[73].

Whether MRI or CT should be the first imaging technique to characterise a nodule after ultrasound depends on the availability and characteristics of either technology in any one centre. Comparison of these is complicated by comparison of different generation machines[74] and different types of tumor. In general modern MRI appears more sensitive for the diagnosis of smaller nodules < 1.5 cm in a cirrhotic liver and in distinguishing regenerative/dysplastic nodules *versus* malignant ones. The importance of the experience of the reporting radiologist should not be underestimated here. Moreover every diagnostic imaging tool has some specific advantage and combination of more than one imaging technique, for difficult focal liver lesions, often increases diagnostic yield.

DIAGNOSIS-LIVER LESION BIOPSY AND TISSUE MARKERS

Many variables affect the accuracy of pathological diagnosis, with sampling being the most important. Many studies evaluating and/or validating immunostaining techniques, have used specimens obtained from resected or explanted livers. However, in clinical practice it is percutaneous biopsy specimens that are available. Biopsy specimens often are small, and represent a fraction of the tumor, so that a lack of immunostaining may simply be the result of inadequate sampling. Using standardized panels with more than one immunostaining method, may result in more confident histological diagnosis of HCC, with a better reproducibility and accuracy, but this needs to be evaluated formally using biopsy specimens.

The role of liver biopsy, in diagnosis of HCC in patients with cirrhosis, has declined during the past few years because better radiological techniques have enormously improved the accuracy of diagnosis. However, when the imaging characteristics are not typical together with AFP < 200 ng/mL, the diagnosis is not reliable without a biopsy sample, as recommended in the joint EASL/AASLD/JSH guidelines[21].

Even though the specificity and positive predictive value of nodule biopsy is high, its negative predictive value is low. Considering that there is a 10% risk of false negativity, the presence of HCC cannot be excluded when a biopsy is negative for HCC. Moreover, it has been suggested that a second biopsy performed immediately after the first one has a limited chance of success, with a gain in diagnosis of HCC of only approximately 5%[44,48,75]. In patients with HCC < 2 cm in whom the first nodule biopsy is negative, repeated imaging is preferred to performing a second biopsy. Whether the risk of false-negative findings is far higher in patients with small nodules (< 2 cm in size), compared with larger nodules, has not been yet clearly demonstrated. However, this is likely, as optimal placement of the needle in smaller nodules is more difficult. Indeed a study of the technical aspects of biopsying nodules ⩽ 1 cm has not been published. However, independent of the size of nodules, the risk of false-negative is higher for those located on the posterior and superior segments of the liver[76].

Percutaneous biopsy of HCC carries a potential risk of tumor seeding along the needle tract. The median risk of seeding is 1%-2%, and is higher if performed alone, compared to combining biopsy with percutaneous ablative techniques[77,78]. Seeding can become manifest after liver transplantation[77,78]. However, if biopsy is necessary, with an indeterminate lesion, it should be combined with percutaneous ablation[77,78]. Another technical variant that may help prevent seeding is the use of a coaxial 17-gauge introducer needle before a 18-gauge biopsy needle, to isolate liver parenchyma during the run of biopsy needle[79]. This should be subject to a comparative study.

Ultrasound-guided fine-needle aspiration (FNA) cytology has been used as an alternative to biopsy because samples can be obtained with smaller needles (23 gauge). Although the specificity and positive predictive value of FNA examination for focal liver lesions is reasonable, the sensitivity ranges between 67% and 93% and thus diagnostic accuracy is less than for histology[77], and risk of seeding is not reduced. Fine-needle aspiration cytology is not recommended for diagnosis.

Histologically diagnosing borderline lesions represents the "Gordian knot" in HCC diagnostics. The three criteria listed from the International Working Party to differentiate HCC from an high grade dysplastic nodule and well-differentiated HCC are: cellular density more than twice normal, irregular nuclear contour of lesion cells and invasion of stroma or portal tract[80]. Tissue

Table 4 Immunohistochemistry for HCC

Marker	Staining pattern	Diagnostic use	Diagnostic value	Ref.
AFP	Specific for HCC Cytoplasm	Expressed in HCC cells cytoplasm but also in: fetal liver, hepatoid tumors, germ cells tumors	Sensitivity 17%-68% Specificity 97% For HCC	[89-104]
GP-3	Specific for HCC Cytoplasm	Expressed in HCC cell cytoplasm (less so if fibrolamellar or sarcomatoid variants) but also in: fetal liver, hepatoblastoma, melanoma	Sensitivity 49%-91% Specificity 89%-100% For HCC	[81,109-115]
CD-34	Endothelium	Surface of normal endothelium and HCC trabeculae or acini but also in: myelodysplasia in transformation, GI stromal tumors (high coexpression with bcl-2)	HCC positivity 82%	[117]
p-CEA	Biliary canalicula	Identifies biliary glycoprotein 1 on hepatocyte canalicular pole and cholangiocyte. Useful for differential diagnosis *vs* cholangiocarcinoma, other adenocarcinoma	HCC positivity 24%-90%	[94,98,100,118-128]
CD-10	Biliary canalicula	Surface of biliary tract cells and in HCC, but also positive in: B cell lymphomas, renal cells carcinoma, melanoma, prostate and pancreas adenocarcinoma. Useful for differential diagnosis *vs* cholangiocarcinoma, other adenocarcinomas	HCC positivity 28%-86%	[94,98,100,119,120,122-124,126,128]
Ki67	Cell proliferation marker	Assessing cell proliferation rate, correlates with tumor grade and clinical course. Useful to differentiate between HCC and hepatic adenoma	HCC positivity rate 10%-50%	[129]
HepPar1	HCC & normal Hepatocyte citoplasm	Expressed in HCC and in normal liver cells, but also in hepatoblastoma. useful for differential diagnosis *vs* cholangiocarcinoma and metastases	HCC positivity 66%-100%	[130-136]
Cytokeratins	Epithelial cells	Useful for differential diagnosis *vs* cholangiocarcinoma. HCC profile: CK7/CK19/CK8/CK18 = - / - / + / +	HCC positivity 76%-96%	[97,101,104,137-140]

markers of HCC might provide a more standardized diagnosis especially for early/well-differentiated HCC and may give information about the probable phenotypic behaviour and thus guide therapy.

Tissue markers

Studies using genome-wide DNA microarray or quantitative real time reverse transcriptase polymerase chain reactions have been done to identify tissue markers of early HCC, in particular to distinguish HCC from dysplastic nodules: Glypican-3 (GPC-3)[81], TGF-b1[82,83], heat shock proteins (HSP) HSP-70 and HSP-27 are the most studied as they inhibit apoptosis[84].

Proteomic studies on liver tissue have traditionally utilized a combination of two-dimensional gel electrophoresis and mass spectrometry analysis[85]. Zeindl-Eberhart *et al*[86] demonstrated the immunoreactivity of aldose reductase-like protein variants (h-ARLP) in cells of HCC and their absence or low signal in normal and cirrhotic livers, fibrolamellar carcinoma or hepatic adenomas. In a more recent study Li *et al*[87] evaluated patients with HCC in HBV-related liver cirrhosis and found 80 proteins with differential expression in HCC. Among these, only two proteins (proliferating cell antigen and stathmin 1) were confirmed by Western blotting analysis.

A 3-gene set (GP3, LYVE1 and survivin) has been proposed as molecular diagnosis of early HCC with accuracy rates of 85%-95% in training and validation sets of more than 70 samples[88].

Current markers available for routine immunochemistry, show different ranges of sensitivity, and few of these are specific to HCC (as are AFP and GP-3). Although there are always false positives and negatives, other markers can be useful for differential diagnosis of HCC *versus* benign nodule or non-HCC cancers, if the specific HCC markers are negative and/or the experience of the pathologist suggests their use. Trying to classify these immunostaining patterns which are useful to build an HCC diagnostic panel, there are five groups of markers (Table 4): 1. specific HCC products, including, AFP and GP-3; 2. vascular pattern markers, such as CD34; 3. canalicular pattern markers, including polyclonal carcinoembryonic antigen (p-CEA) and CD10; 4. markers of cell proliferation such as Ki67; 5. other hepatocellular products as HepPar1 and cytokeratins.

AFP

AFP is a fetal-specific glycoprotein with a molecular weight of 70 kDa, whose synthesis declines rapidly after birth. The immunohistochemical use of AFP staining has very low sensitivity as it detects just 17%-68% of malignant hepatocellular lesions when used alone[89-103]. In a study by Murakata *et al*[104] there was no staining of clear-cell HCC. However, the specificity of AFP is high with an average value of 97%[89,92,93,95,96,99,101,104].

GP-3

GP-3 is a member of the heparan sulfate proteoglycan family, linked to the cell surface through glycosylphosphatidyl inositol, which may also be found in a secreted form. In patients with HCC, GP-3 is over expressed in neoplastic liver tissue and elevated in the serum but is undetectable in normal liver. Melanoma[105], testicular germ cell tumors[106], Wilms tumor, hepatoblastoma[107], among non-HCC malignant neoplasms, and focal

nodular hyperplasia, regenerative and dysplastic cirrhotic nodules, among liver benign and pre-malignant lesions, may be positive for GPC-3[108-110].

Immunostaining for GP-3 can be focal and so it not surprising that there is a lower sensitivity in studies using needle core biopsy[111,112] and FNA[113]. Wang *et al*[109] using 1-mm tissue microarray also demonstrated a sub-optimal sensitivity of GP-3 (70%). In a recent study using a fine-needle aspiration specimen GP-3 had a sensitivity of only 56.8%. The author suggested that the HCC specimen fixation in alcohol rather than in formalin reduced the positivity rate for GP-3[113], although this has not been confirmed. In the literature the GPC-3 sensitivity and specificity ranges from 49% to 90.5% and from 88.5% to 100% respectively[81,109-115]. Dysplastic nodules show a weak positivity of 0%-25% when low grade[109,110,114] and of 20%-75% when high grade[109,110,114].

CD34

CD34 is a 110 kDa transmembrane glycoprotein founded on normal endothelium. It is absent in normal liver sinusoids. The immunostain highlights regions of sinusoids, both in the vicinity of portal tracts in normal liver, and in areas of capillarization such as occur in the periphery of cirrhotic regenerative nodules[116]. In HCC samples an encircling pattern showing the endothelial cells investing the trabeculae and acini or a fine sinusoidal pattern, may be present in 82.5% of cases[117]. Overall specificity of this immunostaining is poor. However, the gradual increase in CD34 expression in HCC, reflecting the progressive arterialization due to hepatocarcinogenesis, eventually results in a complete CD34 immunostaining pattern, so that specificity becomes acceptable[115].

p-CEA

Polyclonal antibodies against carcinoembryonic antigen cross-react with biliary glycoprotein 1 expressed on cholangiocyte surfaces and on hepatocyte canalicular poles. In HCC, biliary glycoprotein 1 co-localisation may show a typical canalicular pattern. This is useful immunostaining for the differential diagnosis of HCC when it is a sclerosing or acinar variant (versus cholangiocarcinoma) or where there is pseudoglandular formation and/or clear cell changes in HCC (usually poorly differentiated HCC which is useful *versus* the differential of epithelial metastases). Immunostaining for p-CEA is reported positive in 24%-90% of HCC[94,98,100,118-128].

CD10

The diagnostic usefulness of CD10 immunostaining is analogous of that of p-CEA, with a lower sensitivity (28%-86%)[94,98,100,119,120,122-124,126,128], but with a specificity of 100%[119,122,123,126,128].

Ki67

Ki67 is a monoclonal antibody which reacts with nuclear proteins expressed in the G1, G2, M and S phases of cell cycle[129]. Evaluating the rate of proliferation is particularly useful for evaluating HCC grade and in differentiating between liver cell adenoma and HCC.

HepPar1

HepPar1 is an antibody which, on paraffin-embedded tissue, links mitochondrial antigens from both malignant and non-malignant hepatic cells, giving a granular cytoplasmic pattern on immunostaining. Is an excellent marker for HCC diagnosis, distinguishing between HCC and liver metastasis or cholangiocarcinoma. It has a similar accuracy to core needle biopsy and in FNA sampling, with a sensitivity of 66%-100% and a specificity of 70.8%-100%[130-136].

Cytokeratins

A subclass of the intermediate filaments family, the cytokeratins (CKs) are expressed in human liver with a characteristic distribution. CK8 and CK18 stain hepatocytes, CK8, CK7, CK18, and CK19 are present in bile duct cells. CK7 and CK19 are not usually present in HCC (76%-96% immunonegative)[97,101,104,137-140], but they exist in many adenocarcinomas, including, cholangiocarcinoma.

DIAGNOSIS-SERUM MOLECULAR MARKERS

Serum tumor markers have several potential uses: for early diagnosis of HCC in high risk patients, in determining prognosis, to estimate tumor volume as well as to monitor therapeutic response and detect recurrence[141].

AFP

The increase of serum AFP may relate to hepatocarcinogenesis but also to hepatic regeneration in chronic liver disease, and may also occur in embryonic carcinomas, gastric and lung cancers[142-147]. Moreover the positive predictive value for the diagnosis of HCC varies with the cut-off value used, ethnicity of the patients, treatment and tumor stage. An AFP with a cut off value of 20 ng/mL has a sensitivity and specificity for HCC diagnosis of 41%-65% and 80%-94% respectively[146]. About 42% of HCC are diagnosed in the absence of a raised AFP[29]. However, AFP confirms the diagnosis of HCC in cirrhosis when the value is over 200 ng/mL when the suspected nodule is larger than 2 cm[21]. A value of $\geqslant$ 400 ng/mL is often associated with the large volume of the tumor mass and/or the carcinomatous involvement of the portal vein, and with a poor median survival rate[145]. In a recent study about 61% of patients with AFP level of > 1000 ng/mL presented with vascular invasion[148].

Alpha-Fetoprotein-L3 (AFP-L3)

Studies on a fucosylated variant of the AFP glycoprotein

which has a high affinity to the sugar chain of Lens culinaris showed increased activities in patients with chronic liver disease and HCC. However, sensitivity and specificity range from 36.5% to 71% and from 63% to 91.6%, respectively[149,150]. When compared with AFP, AFP-L3 showed a higher specificity but similar sensitivity. The disease specificity of this marker is limited as non-tumoral, extrahepatic disease (diabetes, pancreatitis and hypothyroidism) are associated with increased serum levels.

Des-g-carboxy prothrombin (DCP)

DCP also known as a protein induced by the absence of vitamin K or antagonist Ⅱ (PIVKA-Ⅱ) was reported firstly by Liebman *et al*[151] as a candidate biomarker for the diagnosis of HCC. Recent case-control studies documented a sensitivity and specificity ranging from 58% to 89% and 93% to 97% respectively[152-154],and one of these studies found that DCP had a higher sensitivity in the diagnosis of small HCC when compared to AFP and AFP-L3[154].

GPC-3

GPC-3 has been mainly evaluated at the tissue level, although some studies report that GPC-3 can be found in the serum in about 55% of patients with HCC. On the other hand GPC-3 is not detectable in healthy subjects and in patients with liver cirrhosis without HCC[114]. The sensitivity and specificity of serum GPC-3 in diagnosing HCC was reported to be 51% and 90% respectively[155].

GP73

GP73, a Golgi glycoprotein is overexpressed in viral-related HCC[156,157]. Total circulating GP73 can be positive in AFP negative cancer[158].

Other studies have evaluated hepatocyte growth factor[85,159], insulin growth factor[160,161], vascular endothelial growth factor[162] (which correlates with venous invasion), transforming growth factor TGFb1[163-165], IL-6 and IL-10[166,167], hepatoma-specific g-glutamyl-transferase GGTII[168], human cervical cancer oncogene (HCCR)[169] (HCC < 2 cm the positive predictive rate in HCC was 69.2%) and tumor-derived autoantibodies (TAA)[170,171].

Although numerous biomarkers with potential diagnostic or prognostic significance for HCC have been identified there are currently only two FDA-approved tumor markers (AFP and AFP-L3). It is likely that panels of 2 or 3 serum and tissue tumor markers will be used in routine clinical practice in the near future. A standardized approach is required to assess panels of tumor markers and validation is needed in large patient cohorts preferably from multiple centres. In the future, new markers may help not only to diagnose indeterminate lesions based on today's criteria but also to distinguish between HCC with worse or better prognosis.

Diagnosis of HCC lesions in patients with cirrhosis requires a multidisciplinary approach. It follows that specialized centres in which patients may find skilled operators and the most recent diagnostic tools are necessary to optimise diagnosis, particularly with smaller lesions.

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REVIEW

Needle track seeding following percutaneous procedures for hepatocellular carcinoma

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Abstract

Neoplastic seeding may arise after diagnostic or therapeutic percutaneous procedures for hepatocellular carcinoma. The true incidence of seeding with hepatocellular carcinoma is difficult to assess precisely, but a significant risk of seeding exists and is greater when performing diagnostic biopsy as compared to therapeutic percutaneous procedures [radiofrequency ablation, radiofrequency ablation (RFA); percutaneous ethanol injection, Percutaneous ethanol injection (PEI)]. Whenever liver transplantation is feasible, diagnostic needle biopsies should be avoided, but RFA and PEI are often needed as "bridge" treatments. The role of adjuvant treatments in reducing the incidence of seeding following RFA or PEI requires further evaluation.

Key words: Hepatocellular carcinoma; Seeding; Radiofrequency ablation; Percutaneous ethanol injection; Liver biopsy

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a challenging malignancy of global importance. It is the sixth most common cancer, and the third most common cause of cancer-related death worldwide[1]. The incidence rates for HCC in the United States and Western Europe have been increasing[2,3].

Cohort studies and cost-efficiency modelling have suggested that ultra-sonographic surveillance of well-defined cirrhotic patients may decrease tumor-related mortality[4,5]. According to EASL and AASLD criteria[5], a diagnosis of HCC can be made in most cases with a non-invasive strategy. In fact, advances in cross-sectional imaging have led to a drastic limitation in the requirement for biopsy of focal lesions in the cirrhotic liver[6,7]. In this setting, good quality contrast enhanced computed tomography (CT) and magnetic resonance imaging (MRI) are generally highly accurate for the diagnosis of HCC[8,9]. However, biopsy remains very important in patients with a liver nodule without typical radiological features (hypervascularization in arterial phase followed by venous wash-out in portal/venous phase)[5,10], or when α-fetoprotein levels are not diagnostic of HCC (Figure 1).

Although diagnostic biopsy rates have diminished, biopsy is still widely used and is often considered mandatory for patient management by oncologists[11]. Furthermore liver biopsy may give prognostic information[12,13]

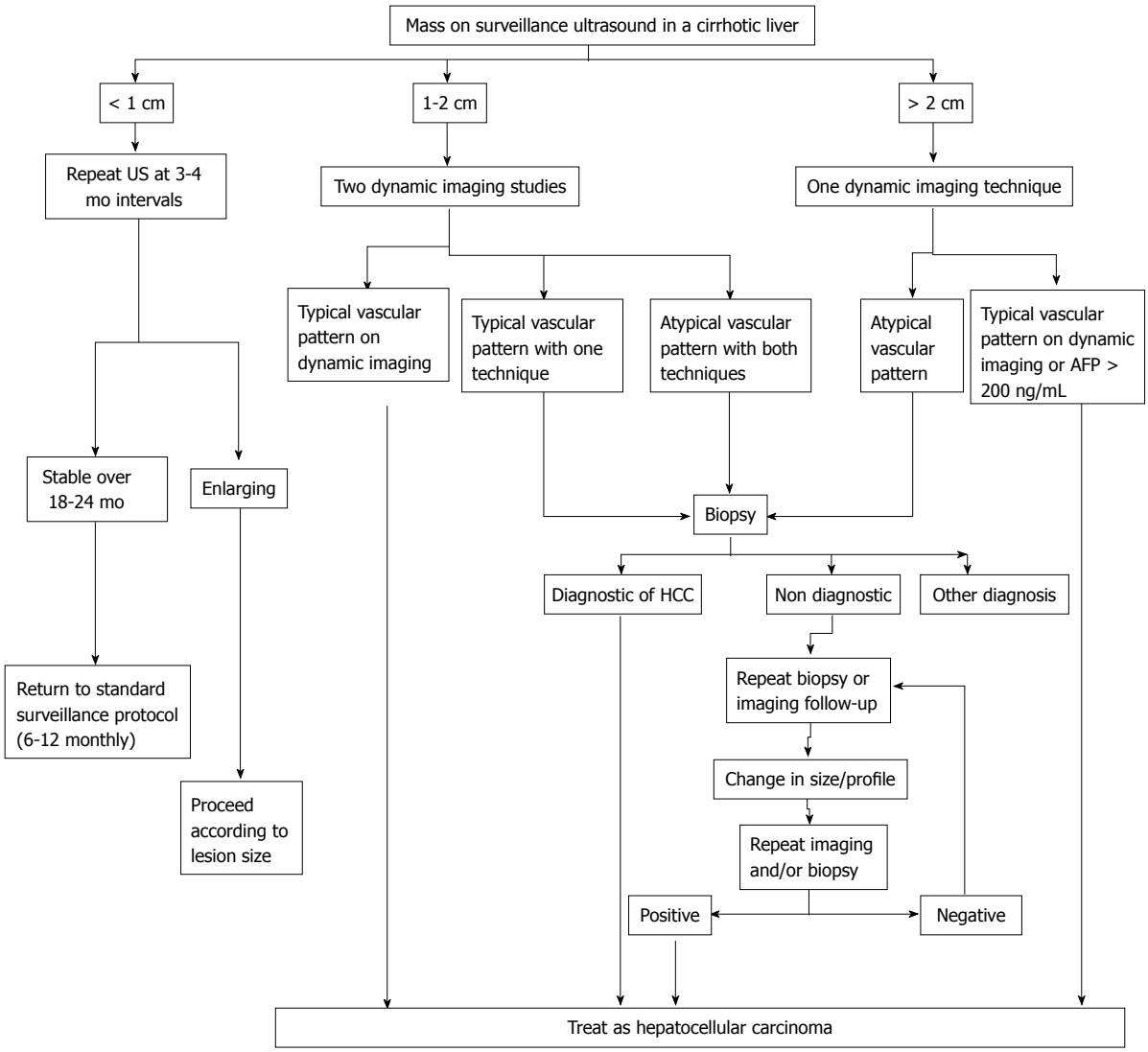

Figure 1 Suggested algorithm for evaluation of a liver mass detected during hepatocellular carcinoma surveillance in a patient with cirrhosis according to the American Association for the Study of Liver Diseases (AASLD) guidelines[5].

regarding malignant potential and may be required for allocation systems in liver transplantation programmes.

Despite many efforts to prevent and screen for HCC, only 20%-30% of patients present with early stage disease amenable to curative treatments, including surgical resection and liver transplantation (LTx)[14]. Radiofrequency ablation (RFA) was introduced as an alternative locoregional therapy to ethanol injection. It can achieve high local cure without deteriorating background liver function. RFA is now a valid alternative to resection, and can be used as an adjuvant therapy, or as a bridge to transplantation[15]. Percutaneous ablation (PEI, RFA) achieves complete responses in more than 80% of tumors smaller than 3 cm in diameter, but only in 50% of tumors between 3 and 5 cm in size[16,17]. Five-year survival rates reported after PEI or RFA are 40%-70%[18,19] RFA is preferred to PEI in patients with tumors < 4 cm. because of lower recurrence, lower number of sessions, and longer disease-free survival. A recent meta-analysis[20] has shown that RFA ablation is superior to PEI in the treatment of small HCC with respect to overall survival, 1, 2, and 3 year survival rates, 1, 2, and 3 cancer-free survival rates, and tumor response. RFA entails a significantly smaller risk of local recurrence than PEI.

NEEDLE TRACT SEEDING

Percutaneous diagnostic or therapeutic procedures play an important role in the management of patients with hepatocellular carcinoma. These procedures are used to diagnose and/or provide specific therapy. All procedures, whether medical or surgical, have inherent risks, and the risks must always be weighed against the anticipated benefits. Both patients and physicians must assume that there is some risk involved when they undertake any procedure including a percutaneous approach for HCC. Complications of percutaneous procedures are sometimes unavoidable. Nonetheless, strict attention to detail and knowledge of potential complications and their risk factors can minimize their occurrence. The incidence of

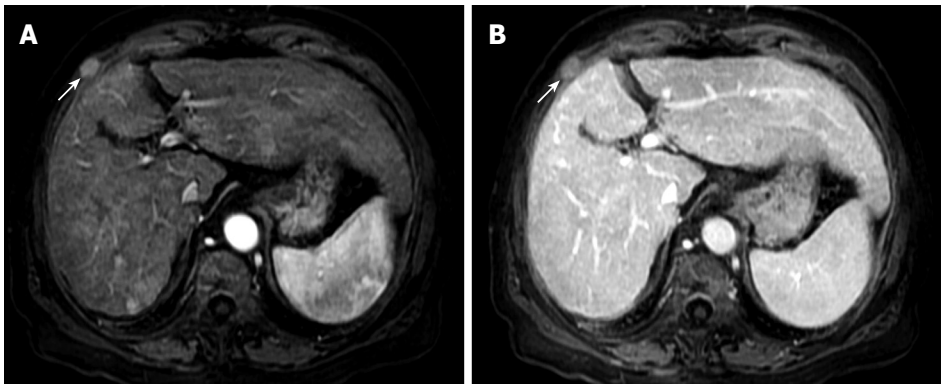

Figure 2 MRI features of HCC seeding following RFA. A: An arterial phase MRI examination of the liver and an enhancing mass in the intercostal space, indicated by the arrowheads, in the right hemithorax; B: A delayed phase MRI examination that shows the mass in the intercostal space, but this is hypointense compared with the rest of the liver.

Table 1 Summary of studies documenting seeding of HCC following biopsy or percutaneous therapeutic techniques in which the cohort size is specified[1]

	Biopsy alone	Biopsy + PEI	RFA	Biopsy + RFA
Series	14	4	5	8
Total patients from series	22424	766	1525	2218
No. seeding from series	43	11	19	24
Risk of seeding mean	3.17%	1.40%	1.73%	2.50%
Risk of seeding median	2.29%	1.40%	0.61%	0.95%
Range	0%-11%	1.15%-1.85%	0%-5.56%	0%-12.5%

[1]Modified from Stigliano *et al*[32]. PEI: Percutaneous ethanol injection of proven HCC; RFA: Radiofrequency ablation of proven HCC.

specific complications is difficult to assess and depends on a number of variables that are not always comparable across studies.

Malignant seeding is a well known complication of both diagnostic and therapeutic procedures in patients with HCC. Needle tract seeding of a liver tumor was previously defined as the development of new neoplastic disease outside the liver capsule, either in the subcutaneous soft tissues of the abdomen, intercostal muscle (Figure 2) or peritoneum[21]. A review[22] identified several suspicious mechanisms that can contribute to seeding after percutaneous procedures: tumor cells may adhere to a biopsy needle or to an electrode during its retraction; tumor cells may also be carried into the track with a little bleeding; furthermore, cells may be forced into the track by sudden increase in intratumoral pressure which is frequently encountered during RFA; finally, cells may be driven in, when saline is injected during or before RFA.

The real incidence of seeding with HCC is difficult to assess precisely. Several studies tried to assess critically the seeding rates after diagnostic and therapeutic procedures, but results are very heterogeneous. Rates of seeding after biopsy range greatly. While modest rates of tumor dissemination of 0.003%-0.009% have been described[23-25], a review on the subject has described incidences of 5%[26]. A recent systematic review and a meta-analysis[27] on needle track seeding following biopsy of liver lesions indicative of HCC showed that the incidence is 2.7% overall [95% Confidence Interval (CI), 1.8-4], and 0.9% per year (95% CI, 0.6-1.3). An important issue of this work is that all diagnosed seedings included in the review, were confirmed histologically.

With increasing use, tumor seeding after percutaneous RFA becomes a major concern, particularly in patients who have a chance of a cure. Llovet *et al*[28] reported a high rate (12.5%) of tumor seeding in 32 patients with HCC treated with RFA which was preceded by a needle biopsy. Neoplastic seeding was associated with subcapsular HCC location, poorly differentiated tumors, and high alpha-fetoprotein levels. Livraghi *et al*[29] reported a low rate of tumor seeding (0.9%) in 1314 patients with a median follow-up of 37 mo, using the same RFA technology as Llovet *et al*[28] and showed that only previous biopsy was significantly associated with tumor seeding. The low risk of seeding after RFA (0%-1.4%) has been confirmed in other recent studies[30]. In a recent study from our group[31], the risk of seeding of hepatocellular carcinoma after radiofrequency ablation was small (1.1% per patient, 95% CI, 0.19-5.84; 0.7% per procedure, 95% CI, 0.12-3.80).

The cause of the high seeding rate reported in the study by Llovet *et al*[28], not confirmed in subsequent studies, remains unclear, but could be due to the shallow position of the nodules (in fact all patients presenting seeding after RFA in the Llovet *et al*[28] cohort had a subcapsular location).

A systematic review[32] of seeding following percutaneous diagnostic and therapeutic approaches for hepatocellular carcinoma, that included studies published as full length papers and as abstracts, founds 179 seeding episodes from January 1983 until February 2007 resulting from diagnostic biopsy or local ablation therapy using percutaneous ethanol injection; acetic acid or hot saline injection; radiofrequency ablation; microwave therapy, laser therapy, or cryotherapy; and combinations of diagnostic biopsy and therapeutic procedures. The mean risk for seeding following diagnostic biopsy was 3.17% (range: 0%-11%). The mean risk for seeding after diagnostic biopsy and percutaneous ethanol injection was 1.4% (range: 1.15%-1.85%). The mean risk for seeding after radiofrequency ablation alone was 1.73% (range: 0%-5.56%). Following biopsy and radiofrequency ablation, the mean risk for seeding was 2.5% (range: 0-12.5) (Table 1).

The time to the diagnosis of seeding ranged from 7 mo for combined percutaneous diagnostic and therapeutic approaches to 13 mo for biopsy alone. From their literature review, the Authors concluded that the risk of the percutaneous approach to HCC is clinically relevant, and that biopsy of HCC in patients with cirrhosis should not be routine clinical practice. Today, there remains no molecular means for classification of HCC. So, we think that suspicious nodule biopsy remains an important research tool to identify new biological variables to achieve a more accurate prognosis of patients with HCC.

RISK FACTORS FOR SEEDING

Numerous factors are related to the risk of neoplastic dissemination after invasive procedures: larger diameter needles[33]; more passes[33]; perpendicular approach[34]; subcapsular tumor location; biopsy prior RFA; intrinsic metastatic properties of the tumor[35] related to either or both tumor size or aggressivity, and patient immunodepression[36].

Poor differentiation of the tumor is a known risk factor for seeding after fine needle aspiration biopsy, and PEI, and is also associated with a higher risk of seeding after RFA[22]. This correlation between seeding and the differentiation of HCC is not clearly evident in the review of Stigliano *et al*[32]. However, a recent study of Imamura *et al*[37] confirms that poor differentiation degree was a risk factor for neoplastic seeding after RFA of HCC and shows that surrogate markers of poor differentiation degree are larger tumor size (> 5 cm) and elevated tumor marker levels (AFP > 100 ng/mL, DCP > 100 mAU/mL, and aAFP-L3 > 15%).

OUTCOME OF SEEDING

In none of the cases of seeding reported in the meta-analyses of Silva *et al*[27] did the seeding event impact on the patient's survival when the lesion was treated successfully by resection and local ablation. Moreover, Livraghi *et al*[29] have reported successful treatment of tumor seeding after RFA by a combination of surgical resection, PEI and RFA and concluded that in no patient did the presence of tumor seeding have a negative impact on survival. Similarly, in the study of Imamura *et al*[37] the cumulative survival rates in HCC patients with neoplastic seeding were 81% at 1 year and 45% at 2 years. The Authors concluded that, taking into consideration the relatively low incidence of neoplastic seeding, the risk of neoplastic seeding after RFA would be considered an acceptable clinical risk.

We have found only one report[38] on malignant seeding after orthotopic liver transplantation, secondary just to pre transplant biopsy. No information about the patient's survival are given.

CONCLUSION

RFA and PEI are associated with a low risk of neoplastic seeding, although the risk of seeding is significantly increased if needle biopsy has been performed before treatment.

Seeding does not have a major negative impact on survival. However, if liver transplantation is a feasible option in the patient's management needle biopsy should be avoided. In fact, assuming a role of the host immune response or of tumor aggressiveness for the low rate of needle seeding, an immunosuppressed condition, as in the post transplant status, could lead to a worsening of survival with a rapid extrahepatic diffusion.

Other strategies, apart from biopsy, such as evaluating new imaging techniques for more precise pre-transplant diagnosis (against explant histology), or simply waiting for a defined period with repeated imaging, may also be effective.

By the same token RFA and PEI, which are often needed as "bridge" treatments for patients on the transplant list, can be used safely without unneeded fears of disseminating the tumor outside of the liver. Additional studies with an adequate duration and quality of follow-up will be paramount to exclude late-onset seeding.

As a future field of research, the incidence of seeding after percutaneous treatments of HCC with new adjuvant treatments such as the antiangiogenic drugs will need to be evaluated.

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REVIEW

Hepatitis C virus and peripheral blood mononuclear cell reservoirs

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Abstract

The existence of hepatitis C virus (HCV) infection in extrahepatic sites has been widely demonstrated. Since peripheral blood mononuclear cells have been the most investigated, compelling evidence of an association with HCV has been shown. Different studies have revealed that HCV RNA can persist and replicate in immune cells but the relevance of its presence and persistence over time is still unknown. As the contribution of this extrahepatic reservoir could have several clinical implications in viral transmission, treatment response and disease pathogenesis, future studies are required to improve our knowledge of the extrahepatic manifestations of HCV and its possible consequences.

Key words: Hepatitis C virus; Peripheral blood mononuclear cell; Reservoirs; Lymphotropism; Replication

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INTRODUCTION

Hepatitis C virus (HCV) is a small positive-strand RNA virus responsible for an important burden of chronic hepatitis and hepatic related diseases around the world[1]. Although HCV is mainly hepatotropic, its presence in extrahepatic sites has been widely demonstrated[2] and it was calculated that the contribution of this second compartment is responsible for about 3.1% of virus in circulation[3]. Lymphoid cells are the most investigated extrahepatic site.

HCV infection of lymphoid cells was suggested for the first time by Hellings in 1985[4]. Mononuclear leucocytes (mainly lymphocytes), isolated by Ficoll-Paque gradient centrifugation of blood freshly drawn from a hemophilia A patient with chronic non-A, non-B hepatitis (NANB), caused NANB when infused in a susceptible chimpanzee.

Immediately after discovery of the virus in 1989[5], different groups attempted to demonstrate HCV replication in lymphoid cells by infecting macrophages, B and T lymphocytes[6-9]. Moreover, several reports describing the presence of the replicative intermediate or negative strand in peripheral blood mononuclear cells (PBMC) were published[9,10]. The HCV RNA negative strand is a viral replicative intermediate and its presence can be considered direct evidence of ongoing viral replication. Nevertheless, discordant results were obtained by different groups, and the association of HCV with PBMC and viral replication in this extrahepatic site remained controversial for many years[11-16]. Strong evidence for *in vivo* HCV infection of, and replication in PBMC was provided by Bronowicki and collaborators[17]. They demonstrated the persistence of the viral RNA sequences in mononuclear blood cells inoculated into immunosup-

pressed mice and they were able to perform a second *in vivo* passage by successful transmission of HCV-RNA-positive cells to other mice. However, later on, the SCID mice did not offer a suitable *in vivo* model to study HCV pathogenesis. Different studies have reported evidence for HCV replication in granulocytes, monocytes/macrophages, dendritic cells, B and T lymphocytes[18-23]. In addition, successful infections of lymphoid cells or establishment of stable HCV+ cell lines have been achieved[22,24].

HCV REPLICATION IN PBMC

As discussed above, the detection of replicative forms of HCV RNA in PBMC has been extensively reported but remains controversial. Earlier PCR methods have been suspected to lack specificity and/or sensitivity, possibly due to the very low concentration of negative HCV RNA strand in cells. Currently, methodological modifications have been used to overcome these difficulties and many reports demonstrated that HCV can certainly replicate in PBMC[25-29].

Some studies showed that replication in PBMC occurs at a very low level and the amount of intracellular HCV RNA is patient-specific and is a result of a dynamic process related to virologic and immunologic factors[26,28]. The role of HCV lymphotropism in the natural history of HCV infection is not yet resolved and reports remain arguable. Nowadays, although it is accepted that HCV can replicate in PBMC, the contribution of this extrahepatic site as a significant viral reservoir and the importance of viral persistence in aviremic subjects after spontaneous or therapeutical clearance is still under debate.

PBMC AS HCV RESERVOIRS

Lymphoid cells may represent privileged reservoirs that could favor HCV persistence leading to chronic HCV infection. The infection of immune cells may interfere with the efficiency of viral clearance by the host[30,31]. Different reports demonstrate the persistence of HCV RNA at very low levels in serum and peripheral lymphoid cells after apparently complete spontaneous or antiviral therapy-induced resolution of chronic hepatitis C[32,33]. The occult HCV persistence in lymphoid cells may have important epidemiological and pathogenic implications. Radkowski and collaborators[33] suggested that in patients with sustained virological response (SVR), small quantities of HCV RNA may persist in liver or PBMC for up to 9 years. These findings could explain the phenomenon of frequent persistence of humoral and cellular immunity for many years after supposed viral clearance but also, could present a potential risk for transmission and reactivation. It was also demonstrated that HCV may also persist and replicate in the liver and PBMC of healthy, anti-HCV antibody-positive, serum HCV RNA-negative patients who have persistently normal ALT levels[34]. It is possible that viral persistence and, specifically, the presence of HCV RNA in PBMC may lead to HCV reactivation under special circumstances. In patients with immunosuppresion, under immunomodulatory therapy or with coinfection, persistent replicating HCV could represent a potential source for viral recurrence. These findings suggest that sterilizing immunity with complete elimination of virus is unlikely.

EVIDENCE AGAINST PBMC AS LONG-LIVED HCV RESERVOIRS

In contrast with the above mentioned reports, some studies refute the role of PBMC as a long-lived HCV reservoir. Kaiser P and collaborators[26] evaluated 30 HIV/HCV coinfected patients for up to 40 mo. Total PBMC-associated HCV RNA and virion-enclosed PBMC-associated HCV RNA, that could represent viral particles nonspecifically attached to blood cells, were distinguished and they observed widespread presence of viral RNA in PBMC from HCV-viremic patients. Evidence for persistence of HCV in PBMC in the absence of HCV viremia in plasma could not be found. Their experiments supported a concept of low level replication in PBMC and suggested that the infection and expression of HCV in PBMC is of minor quantitative importance for systemic replication and persistence of HCV[26].

Another report in which HCV persistence was underestimated was published by Bernardin[35]. In their experiments, they could not find any HCV RNA detectable PBMC sample in 69 aviremic donors indicating that PBMC are unlikely to serve as a long-lived reservoir of HCV in aviremic subjects.

The slow decrease in anti-HCV antibody titers in subjects with spontaneously cleared viremia as well as the complete seroreversion detected in 7% of transfusion transmitted infections may also reflect an absence of ongoing antigenic stimulation, indirectly supporting clearance of infection in persons who test HCV RNA-negative in plasma[35].

HCV LYMPHOTROPISM

The presence of variants of the highly conserved 5' untranslated region (UTR) have been observed between HCV from plasma and PBMC[36,37]. The identification of sequence polymorphisms in cells of the lymphatic system suggested possible adaptation of HCV to replicate in nonhepatic cells[29]. In addition, a compartmental distribution of HCV quasispecies and HCV genotypes has been demonstrated[37,38]. Concordant with our results in a hemophilic population, the HCV genotypes detected in PBMC were not detected in plasma in some individuals supporting independent replication in these cells[37,39].

These findings further support the lymphotropic nature of HCV and reinforce the concept that independent replication of HCV in lymphoid cells may constitute a potential risk for persistence, reactivation, recurrence or treatment resistance.

CLINICAL IMPLICATIONS OF PBMC AS HCV RESERVOIRS

HCV transmission

In some contexts, as in vertical transmission, the presence of HCV in PBMC played relevant roles. The risk of mother-to-child transmission is associated with the presence of HCV in maternal PBMC[40]. Likewise, the persistence of small quantities of HCV RNA in aviremic patients who are supposed to have solved the infection could have important implications for viral transmission.

Reactivation or recurrence

Several studies demonstrate that relapse after sustained virological response is extremely rare[41]. However, low-level intrahepatic viraemia despite negative serum HCV RNA testing has been shown to predict a higher likelihood of late relapse, particularly in the setting of immunosuppression[41].

The existence of PBMC reservoirs may be implicated in the recurrence of chronic hepatitis after apparently successful antiviral treatment. Previous findings suggest that in patients with spontaneous eradication or sustained virological response after therapy, small quantities of HCV RNA may persist in lymphoid cells for years[32,33]. The presence of positive/negative strand HCV RNA at the end of treatment was associated with relapse among HCV-HIV coinfected patients[42]. It has been suggested that low level replication of HCV in PBMCs may lead to reactivation of HCV after termination of therapy[2,32,33].

Reemergence of HCV RNA was demonstrated in apparent sustained viral responders receiving immune suppressive therapy[43,44]. This proved that the HCV reservoir requires continued innate or T cell immune surveillance to prevent disease activity even years after the infection in at least some sustained viral responders[43].

On the other hand, recurrent infection in transplant recipients was also described[45-47] and the utilization of antiviral therapy in HCV-infected patients awaiting liver transplantation as one of the strategies to prevent hepatitis C recurrence after transplantation was recommended[48]. It has been proposed that viral variants from extrahepatic compartments may be involved in infection recurrence after liver transplantation. One report has specifically investigated the origin of HCV recurrence and suggested that liver-derived virus remaining in circulation was the major responsible for the graft reinfection. However, virus variants of likely extrahepatic origin could be detected in serum early after transplantation[45].

Immune dysfunction and lymphoproliferative disorders

The interaction between HCV and the human immune system is likely to have important clinical consequences. First, HCV has a remarkable ability to evade the immune system, achieving almost 85% chronicity rates. On the other hand, HCV infection may induce extra-hepatic immune related manifestations in a high percentage of infected patients, including mixed cryoglobulinemia and non-Hodgkin lymphoma. At present, the possible mechanisms by which HCV modulates immune function are being examined.

Monocytes, B cells, and CD4+ and CD8+ lymphocytes can support HCV replication and can serve as reservoirs in symptomatic and occult HCV infections[29]. Endogenous presentation of HCV antigens by infected B cells and monocytes may contribute to immune tolerance of HCV, favoring its persistence[29]. Particularly, in perihepatic lymph nodes, HCV replication has been demonstrated and the results suggest that replication of HCV in T cells might contribute to disturbance of Th1 commitment or Th1 hyporesponsiveness in individuals with persistent HCV infection[31].

According to some reports, a predominant infection of B lymphocytes suggests a preferential tropism of HCV for B cells[49,24]. In consequence, chronic antigen stimulation by the virus may trigger B cell proliferation resulting in a wide spectrum of lymphoproliferative disorders, cryoglobulinemia and non-Hodgkin lymphoma, frequently observed in infected patients[2,50].

Treatment resistance

Different factors have been associated with lower treatment response, such as higher serum HCV viral load at baseline, HCV genotype 1 infection, co-treatment with antiretroviral therapy, the presence of IFN-neutralizing antibodies and a higher degree of immune deterioration[51-56]. The detection of HCV RNA in PBMC reservoirs might have important implications for effective treatment. One possible mechanism of relapse is that PBMC could serve as a viral reservoir resistant to IFN[30]. As demonstrated, clearance of HCV RNA in PBMC at the end of IFN treatment was a predictor of durable response to antiviral therapy in patients with chronic hepatitis[57]. Moreover, in the context of HCV-HIV coinfected patients, the presence of strand specific HCV RNA at the end of 48 wk of therapy was associated with viral relapse[41].

On the other hand, HCV lymphotropic variants corresponding to genotypes more resistant to treatment could persist in lymphoid cells[58,39]. Interestingly, Di Liberto and collaborators found that the presence of compartmentalization in PBMC was strongly predictive of sustained virologic response[59].

CONCLUSION

To date, different reports suggest that HCV replication in PBMC does definitely occur which proved to have important effects on different aspects of HCV infection. Nonetheless, as conflicting findings *in vivo* still exist, the contribution of this extrahepatic site in disease pathogenesis and treatment should be further explored and the mechanisms involved should be elucidated.

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REVIEW

Oxidative stress signaling underlying liver disease and hepatoprotective mechanisms

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Abstract

Oxidative stress is a redox imbalance between pro-oxidants and antioxidants in favour of the former ones, leading to different responses depending on the level of pro-oxidants and the duration of the exposure. In this article, we discuss the damaging or cytoprotective signaling mechanisms associated with oxidative stress by addressing (1) the role of prolonged and severe oxidative stress and insulin resistance as determinant factors in the pathogenesis of non-alcoholic fatty liver disease associated with obesity, which, with the concurrence of nutritional factors, may determine the onset of fatty liver and its progression to steatohepatitis; and (2) the development of an acute and mild pro-oxidant state by thyroid hormone administration, which elicits the redox up-regulation of the expression of proteins affording cell protection, as a preconditioning strategy against ischemia-reperfusion liver injury.

Key words: Oxidative stress; Obesity; Insulin resistance; Non-alcoholic fatty liver disease; Thyroid hormone; Liver preconditioning

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INTRODUCTION

Oxidative stress is a redox disequilibrium in which the pro-oxidant/antioxidant balance is shifted in favour of the pro-oxidants[1], a phenomenon related to the aerobic nature of cellular metabolism, in which O_2 reduction is a major event. The latter proceeds through electron transfer reactions due to the electronic structure of O_2 in the ground state, with generation of reactive oxygen species (ROS), including (1) primary oxidants [superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$)]; and (2) secondary oxidants [hydroperoxides or alkoxy and peroxy radicals of biomolecules, in addition to electronically excited states derived from free-radical reactions (singlet oxygen, triplet carbonyls)][2]. The detoxication of ROS is a major prerequisite of aerobic life[1], which is accomplished *via* several enzymatic and non-enzymatic antioxidant mechanisms that are available in different cell compartments[1,3]. Secondary mechanisms, restoring used cofactors and repairing altered biomolecules, are also required, in addition to those triggering the expression of proteins damaged by ROS or needed to attain cell survival[1,3,4]. These mechanisms need to be coupled to the intermediary metabolism for ATP, NADPH, and precursors supply, and depend on the dietary replenishment of essential components to maintain pro-oxidant reactions and cellular damage at a minimum level under basal conditions.

At the cellular level, oxidative stress leads to a wide

spectrum of responses, depending on the cell type, the level of ROS achieved, and the duration of the exposure[4-6]. The moderate increase in ROS and reactive nitrogen species (RNS) in a defined time window can elicit an imbalance capable of redox regulation, as found for L-3,3',5-triiodothyronine (T_3)-induced oxidative stress[7], involving important signals regulating either protein function, *via* reversible oxidation or nitrosation of protein sulfhydryls, and/or gene expression, through modulation of specific kinases, phosphatases, and redox-sensitive transcription factors[4-6]. However, in the case of organs subjected to ischemia-reperfusion (IR)[8] or in obesity[9] and other chronic states, large levels of ROS are attained, which may induce severe oxidation of biomolecules and dysregulation of signal transduction and gene expression, leading to cell death through necrotic and/or apoptotic mechanisms[4].

In this review article, the damaging or cytoprotective signaling mechanisms associated with oxidative stress are addressed. In particular, I will discuss (1) the role of oxidative stress and insulin resistance as contributing factors in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) in obese patients, which, with the concurrence of nutritional factors, may determine the onset of fatty liver and its progression to steatohepatitis; and (2) the implications of the redox regulation of T_3-induced gene expression as a preconditioning mechanism against IR liver injury.

OXIDATIVE STRESS SIGNALING UNDERLYING OBESITY-ASSOCIATED NAFLD

The onset of oxidative stress, insulin resistance, and steatosis in obese NAFLD patients

NAFLD is a rapidly growing entity that is becoming a major cause of chronic liver disease, due to the increasing incidence of obesity and type 2 diabetes in the general population. NAFLD includes simple triacylglycerol (TAG) accumulation in hepatocytes (hepatic steatosis) or steatosis with inflammation, fibrosis, and cirrhosis (non-alcoholic steatohepatitis, NASH), with oxidative stress, insulin resistance, and nutritional factors playing major contributing roles[10,11].

Under most circumstances, fatty acids (FA) are the major oxidative fuel in the liver. However, carbohydrate and lipid affluence induce significant changes in hepatic intermediary metabolism. In fact, high glucose and insulin levels stimulate FA synthesis from glucose and inhibit FA β-oxidation, re-directing FA towards the formation of TAG[9]. Considering that the amount of TAG exported as VLDL depends on synthesis of the protein components, FA in excess are likely to be converted to TAG and stored as lipid droplets within hepatocytes, upon consumption of calorie-enriched diets. Since non-adipose tissues have limited capacity for TAG storage, the lipids in excess that accumulate under conditions of overnutrition determine high intracellular levels of saturated FA, which can induce cell dysfunction and/or cell death, a phenomenon known as lipotoxicity[12]. Consequently, higher rates of FA oxidation and ROS generation are achieved, which might explain the increase in the oxidative stress-related parameters and antioxidant depletion found in the liver of obese patients with NAFLD (Figure 1)[9,13]. Furthermore, prolonged oxidative stress may favour: (1) liver *n-3* LCPUFA depletion, which may be compounded by dietary imbalance and defective desaturation activity[14,15]; and (2) insulin resistance, in association with the redox activation of multiple stress-sensitive serine/threonine kinases that alters insulin signaling (Figure 1)[16]. The latter phenomenon is a membrane-mediated process that might be also compromised by *n-3* LCPUFA depletion, due to loss of membrane polyunsaturation. Both IR and liver *n-3* LCPUFA depletion can determine hepatic steatosis by different mechanisms, namely, (1) insulin resistance-dependent higher peripheral mobilization of FA and glycerol to the liver; and (2) *n-3* LCPUFA depletion-induced changes in the DNA-binding activity of the peroxisome proliferator-activated receptor-α (PPAR-α) as well as of the sterol regulatory element binding protein-1c (SREBP-1c), determining a metabolic imbalance between FA oxidation and lipogenesis in favour of the latter (Figure 1). This notion is based on the findings that *n-3* LCPUFA are signaling biomolecules regulating hepatic lipid metabolism through (1) down-regulation

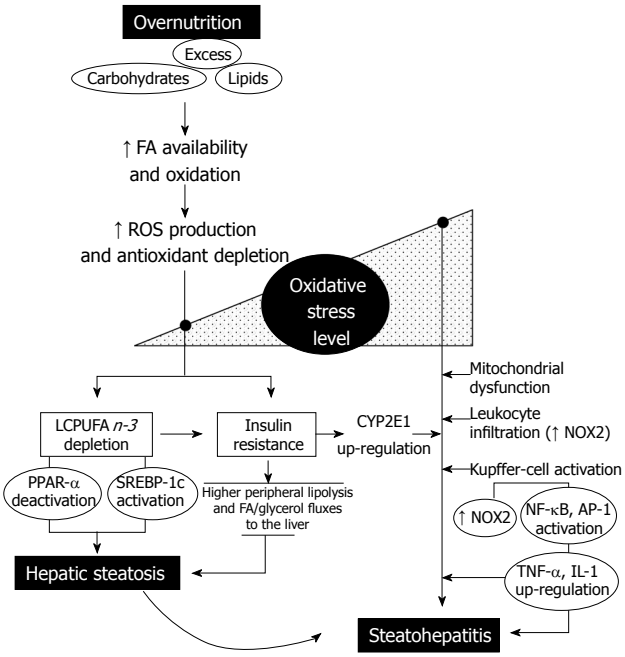

Figure 1 Interrelationships between the level of oxidative stress and insulin resistance, leading to hepatic steatosis and its progression to steatohepatitis, associated with overnutrition. AP-1: Activating protein 1; CYP2E1: Ethanol inducible form of cytochrome P450; FA: Fatty acids; IL-1: Interleukin-1; LCPUFA; Long-chain polyunsaturated fatty acids; NF-κB: Nuclear factor-κB; NOX2: NADPH oxidase in phagocytic cells; PPAR-α: Peroxisome proliferator-activated receptor-α; ROS: Reactive oxygen species; SREBP-1c: Sterol regulatory element binding protein-1c; TNF-α: Tumor necrosis factor-α.

of the expression of SREBP-1c and its processing, with inhibition of the transcription of lipogenic and glycolytic genes; and (2) up-regulation of the expression of genes encoding enzymes of the oxidation of FA, which act as ligands of PPAR-α[17].

Exacerbation of hepatic oxidative stress and progression from steatosis to steatohepatitis

Changes in liver oxidative stress-related parameters observed in obese patients with steatosis persist in those with steatohepatitis[9,13]. In steatohepatitis, these features are observed concomitantly with (1) low catalase activity[13]; (2) high immunohistochemical reactivity to 8-hydroxydeoxyguanosine and 4-hydroxy-2-nonenal, as markers of oxidative DNA damage and lipid peroxidation, respectively[18]; (3) a further increment of both 3-nitrotyrosine immunoreactivity and production of $O_2^{\bullet-}$ and malondialdehyde by Kupffer cells; (4) induction of inducible nitric oxide synthase; and (5) up-regulation of cytochrome P450 2E1 (CYP2E1), as shown by the higher CYP2E1 protein expression and *in vivo* chlorzoxazone hydroxylation, an indicator of CYP2E1 activity (Figure 1) (for specific references see[9]).

The exacerbation of the oxidative stress status of the liver in cases of steatohepatitis, compared to livers with steatosis alone, seems to involve several mechanisms (Figure 1). First, induction of liver CYP2E1[13,19] is of particular importance in the pathogenesis of NASH, due to its poor coupling with NADPH-cytochrome P450 reductase, with substantial NADPH oxidase activity, leading to $O_2^{\bullet-}$, H_2O_2, and consequent lipid peroxidation[20]. Second, hepatic mitochondrial dysfunction is an alternate contributing factor to the genesis of lesions in steatohepatitis, considering the lower levels of mitochondrial DNA and the decreased expression of mitochondrial DNA-encoded proteins, which might lead to reduced activity of respiratory complexes Ⅰ, Ⅲ, and Ⅳ, and ATP synthase complex Ⅴ, thus increasing $O_2^{\bullet-}$ and H_2O_2 generation[10]. Third, mixed inflammatory-cell infiltration is a characteristic feature of NASH, including mononuclear cells, polymorphonuclear cells, or both[10], which may represent an additional mechanism of ROS generation, due to the expression and activation of NADPH oxidase (NOX2), an enzyme that produces large amounts of $O_2^{\bullet-}$ and H_2O_2[21]. NOX2 is also expressed in Kupffer cells, which, in patients with steatohepatitis, produce $O_2^{\bullet-}$ at rates that are 20-fold higher than normal, in agreement with the 7-fold increase in malondialdehyde levels in Kupffer cells from steatohepatitis patients[22]. Under these conditions, the oxidative stress status of the liver achieved in steatohepatitis might promote hepatocellular damage by inducing (1) severe oxidative alteration of biomolecules, with loss of their functions and impairment of cell viability; and (2) sustained activation of redox-sensitive transcription factors, such as NF-κB and AP-1, with consequent up-regulation of the expression of pro-inflammatory mediators at the Kupffer cell level (Figure 1)[23].

Collectively, the discussed evidence supports the view of a functional interdependence between oxidative stress and insulin resistance (Figure 1). This may involve (1) initial ROS production due to lipotoxicity, related to the onset of insulin resistance in steatosis; and (2) a further increase in ROS generation due to CYP2E1 induction, mitochondrial dysfunction, and Kupffer cell or infiltrating leukocyte NOX2 activity, characterizing steatohepatitis. Dysregulation of pro-inflammatory cytokine, adipokine, and chemokine signaling in NAFLD may reinforce the initial mechanisms of ROS production and IR, representing key factors in the progression from steatosis to steatohepatitis, in the setting of oxidative stress-mediated hepatocyte sensitization[11,24]. In this context, antioxidants can act as insulin sensitizers by lowering ROS levels, a condition that might abrogate free-radical-mediated activation of signaling serine/threonine kinases and damage to biomolecules, as shown in cell-culture studies[25]. However, these findings remain to be confirmed in obese patients with NAFLD.

OXIDATIVE STRESS SIGNALING UNDERLYING THYROID HORMONE LIVER PRECONDITIONING

Mechanisms in thyroid hormone calorigenesis and liver oxidative stress

Thyroid hormones play important roles in cell growth, differentiation, and metabolism, through different and complex mechanisms of action. In mammals, major effects are exerted on cellular oxygen consumption (QO_2) and metabolic rate, leading to stimulation and maintenance of basal thermogenesis[26,27]. This action of T_3 is carried out *via* thyroid hormone receptors expressed in almost all tissues. These receptors are recognized by specific thyroid hormone response elements across the DNA, leading to ligand-dependent upregulation of the expression of respiratory, metabolic, and uncoupling protein genes (Figure 2A)[28]. In addition to the above classical genomic model of T_3-dependent calorigenesis, non-genomic mechanisms may also contribute to increase cellular QO_2[29], with the consequent increase of the mitochondrial capacity for oxidative phosphorylation and ROS generation[30].

In addition to T_3-induced liver mitochondrial capacity for ROS production, the induction of other enzymatic mechanisms also occurs, namely, (1) higher activity of microsomal NADPH-cytochrome P450 reductase[31] and NADPH oxidase[32], the latter representing the oxidase activity of cytochrome P450 responsible for the $O_2^{\bullet-}$ and H_2O_2 production related to the T_3-mediated induction of the highly pro-oxidant cytochrome P4502E1 isoform[33]; (2) enhancement of cytosolic enzymatic mechanisms, such as the $O_2^{\bullet-}/H_2O_2$ generator xanthine oxidase[34] and ROS production, possibly coupled to enhanced FA β-oxidation due to liver peroxisomal proliferation[30]; and (3) Kupffer-cell activation with increased respiratory

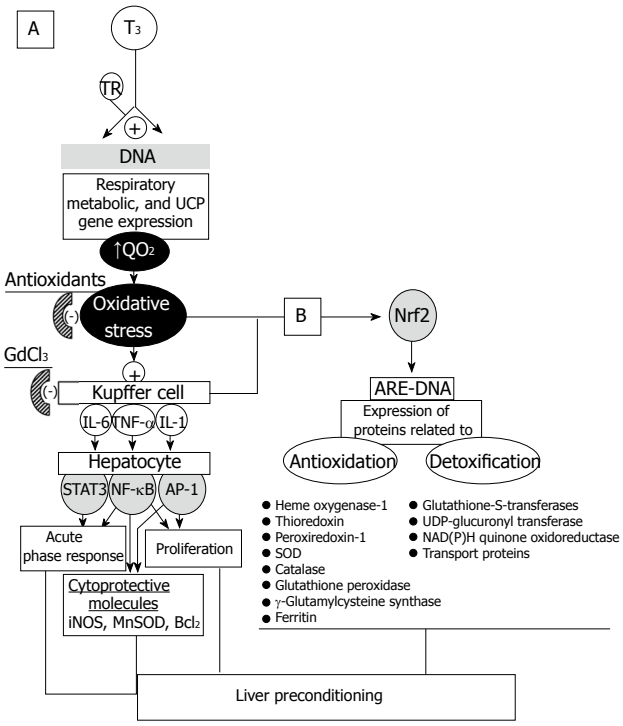

Figure 2 Oxidative stress signaling in thyroid hormone (T3) liver preconditioning as mediated by redox-sensitive transcriptional factors NF-κB, AP-1, and STAT3 (A) or Nrf2 (B). AP-1: Activating protein 1; ARE: Antioxidant responsive element; GdCl3: Gadolinium chloride; IL: Interleukin; iNOS: Inducible nitric oxide synthase; MnSOD: Manganese superoxide dismutase; NF-κB: Nuclear factor-κB; Nrf2: Nuclear factor-erythroid 2-related factor 2; QO2: Rate of oxygen consumption; TNF-α: Tumor necrosis factor-α; STAT3: Signal transducer and activator of transcription 3; TR: Thyroid hormone receptor; UCP: Uncoupling protein.

burst activity, due to NADPH oxidase[35].

T_3-induced liver free-radical activity is associated with depletion of antioxidant defences, leading to increased oxidative stress of the liver (Figure 2A)[7,28,36]. However, this pro-oxidant state achieved in the liver by T_3-induced calorigenesis can be considered as a mild redox alteration, as suggested by the lack of occurrence of morphological changes in liver parenchyma, except for the significant hyperplasia and hypertrophy of Kupffer cells[35], the resident macrophages of the liver[37]. The latter effect of T_3 might be of importance considering that Kupffer cells play a central role in the homeostatic response to liver injury, through the production and release of a wide array of mediators that provide physiologically diverse and key paracrine effects on all other liver cells[37,38].

T_3-induced Kupffer cell-dependent up-regulation of cytokine expression and hepatocyte proteins related to antioxidation, anti-apoptosis, acute-phase response, and cell proliferation

Kupffer cell hyperplasia is a major finding after *in vivo* T_3 administration, an effect that may involve the expansion of Kupffer cell precursors by means of circulating monocyte recruitment, the differentiation of pre-existing local Kupffer cell precursors into mature liver macrophages, or both[39]. Under these conditions, assessment of Kupffer cell function revealed a significant increase in the rate of carbon phagocytosis and the associated carbon-induced O_2 uptake, representing the respiratory burst activity of Kupffer cells, a process that is largely dependent on the activity of the ROS-generator NADPH oxidase and abolished by pretreatment with the Kupffer cell inactivator gadolinium chloride ($GdCl_3$) (Figure 2A)[35].

The interdependence between T_3-induced calorigenesis, liver QO_2, and ROS production is associated with a significant increase in the hepatic DNA binding of the transcription factors NF-κB[40], STAT3[41], and AP-1[42] (Figure 2A). Activation of these transcription factors by T_3 administration is suppressed by *in vivo* pretreatment with $GdCl_3$, whereas NF-κB and STAT3 activation by T_3 is also abolished by pretreatment with antioxidants[40,41], thus supporting the view that T_3 induces the redox activation of hepatic NF-κB, STAT3, and AP-1 by actions primarily exerted at the Kupffer cell level (Figure 2A). T_3 administration involving significant NF-κB and AP-1 activation induced mRNA expression of the NF-κB/AP-1-responsive genes for TNF-α, with increased serum levels of the cytokine[40] that are abolished by pretreatment with the antisense oligonucleotide TJU-2755, targeting the primary RNA transcript of TNF-α[43]. T_3 also elicited an increase in the serum levels of IL-6[41] and in the hepatic mRNA expression and serum levels of IL-1[40]. In addition to NF-κB and AP-1 activation, the enhancement in STAT3 DNA binding by T_3 administration[41] may be associated with the proliferation of macrophage precursors and their differentiation into Kupffer cells[39], considering the central role of STAT3 in gp130-mediated cell growth, differentiation, and survival[44].

The effects of cytokines released from Kupffer cells are exerted through their interaction with specific surface receptors of liver target cells, mediating the signaling transduction from the cell membrane to the nucleus[37]. In agreement with the above view, the transient TNF-α response elicited by T_3 administration correlates with the substantial increase in liver IκB-α phosphorylation[45,46], leading to the activation of the IKK complex that in turn activates NF-κB, after coupling with the TNF-α receptor and associating with different adaptor proteins[47]. T_3-induced TNF-α response, liver IKK phosphorylation, and NF-κB activation are abolished by pretreatment with either α-tocopherol or $GdCl_3$, supporting the role of ROS production and Kupffer-cell activation in T_3-dependent signaling leading to up-regulation of hepatic gene expression[45,46]. This is shown by the increased expression of the NF-κB-responsive genes encoding for inducible NOS (iNOS)[45], manganese superoxide dismutase (MnSOD), and the anti-apoptotic protein Bcl-2[46] (Figure 2A). Thus, T_3 administration elicits the redox up-regulation of iNOS, MnSOD, and Bcl-2 in the liver, in association with the Kupffer cell-dependent release of TNF-α and activation of the IKK/NF-κB cascade, representing antioxidant and anti-apoptotic responses triggered by the underlying oxidative stress (Figure 2A).

In addition to the above responses, T_3 administration up-regulate the acute-phase response (APR) of the liver and the hepatocyte proliferation. The APR is a major pathophysiologic reaction in which normal homeostatic mechanisms are replaced by new set-points, contributing to defensive or adaptive capabilities against inflammation and oxidative stress[48,49]. In fact, T_3 induced the Kupffer-cell-dependent release of IL-6 and activation of hepatic STAT3 controlling both type I (haptoglobin) and type Ⅱ (β-fibrinogen) acute-phase protein (APP) genes[41]. In addition, this response may be contributed by the T_3-induced TNF-α/IKK/NF-κB pathway[45,46], which controls type I APP genes, considering that NF-κB activation can synergistically enhance the effects of STAT3 and C/EBPβ upon C-reactive protein induction[50]. Furthermore, the *in vivo* effects of T_3 as a primary hepatic mitogen, leading to hepatocyte proliferation in intact liver, are well established (Figure 2A)[42]. This process involves a large number of genes and requires the concurrence of cytokines, growth factors and metabolic networks[51]. Resting hepatocytes, i.e. in the G0 phase of the cell cycle, need to be primed by TNF-α and IL-6 before they can respond to growth factors, with the concomitant activation of NF-κB, STAT3, AP-1, and E/EBPβ, enter the G1 phase and initiate cell cycle progression. T_3 administration has been associated with increased liver cyclin-dependent kinase 2 expression and hepatocyte proliferation, as shown by the increase of Ki-67, a nuclear cell proliferation-associated protein expressed in all active parts of the cell cycle, and of the proliferating cell nuclear antigen (PCNA)[42].

Collectively, data reported by our group indicate that T_3 triggers cytoprotection in the liver through redox- and Kupffer cell-dependent signaling mechanisms, namely, (1) antioxidant responses (iNOS, MnSOD); (2) anti-apoptosis (Bcl-2); (3) immune, transport, and antioxidant (haptoglobin, ceruloplasmin, ferritin) functions fulfilled by APR induction; and (4) hepatocyte proliferation (Figure 2A), the metabolic demands of which being met by acceleration of energy metabolism due to T_3-induced calorigenesis[7,28,52].

Thyroid hormone-induced liver preconditioning

Organ preconditioning, including that involving the liver, consists in strategies protecting the organ from detrimental effects of subsequent noxious events, such as those underlying chemically-induced injury or IR[8,53]. In general terms, IR injury refers to tissue damage produced by blood perfusion to a previously ischemic organ. In the case of the liver, this occurs in the clinical settings of hepatic resection, transplantation, low-blood pressure states, and abdominal surgery requiring hepatic vascular occlusion. IR liver injury assessed in a model involving 1 h of partial ischemia, as induced by vascular clamping, and followed by reperfusion for 20 h, elicited minimal mortality but substantial liver damage, with increased serum transaminase and TNF-α levels as well as metabolic changes, namely, (1) a drastic increase in the oxidative stress status of the liver; (2) loss in the DNA binding of NF-κB and STAT3, implying loss of cytoprotective potential, as shown by the concomitant diminution in the expression of the APR protein haptoglobin, controlled by both these transcription factors; and (3) increase of the hepatic AP-1 DNA binding activity, which may constitute a major determinant of hepatotoxicity under conditions of reduced NF-κB activation and TNF-α response[54,55]. These changes were normalized by T_3 treatment given 48 h before the IR protocol, a preconditioning effect that was sensitive to the antioxidant N-acetylcysteine given prior to T_3[55], with enhanced hepatocyte proliferation compensating for liver cells lost due to IR-induced hepatocellular necrosis[42].

In conclusion, the data discussed above indicate that redox regulation of gene transcription by T_3 involves antioxidant-sensitive NF-κB, AP-1, and STAT3 activation and up-regulation of the expression of cytoprotective proteins affording liver preconditioning (Figure 2A)[52]. T_3 liver preconditioning may also involve the activation of the Nrf2-Keap1 defense pathway, up-regulating antioxidant proteins and phase-2 detoxifying enzymes (Figure 2B)[56], which is currently under study in our laboratory.

CONCLUSION

Data analyzed indicate that development of extreme levels of oxidative stress in the liver determines opposite cellular responses, depending on the period of exposure to ROS. Development of a progressive and severe pro-oxidant state in the liver of obese patients with NAFLD is associated with the onset of steatosis and its progression to steatohepatitis, as a chronic model of nutritional oxidative stress. The molecular pathogenesis of NAFLD in obese patients seems to be multifactorial, with oxidative stress and insulin resistance as major pathophysiological mechanisms, which may be interdependent[24]. Considering the lack of an effective drug therapy for NAFLD at present[10], further studies of potentially attractive therapeutic targets are required. These may include the expression and activation status of metabolic (PPAR-α and SREBP-1c) and pro-inflammatory (NF-κB and AP-1) transcription factors, and the activity of enzymes associated with insulin resistance, such as serine/threonine stress kinases and protein tyrosine phosphatases, which will undoubtedly contribute to understand the role of chronic and progressive oxidative stress and insulin resistance in determining steatosis and its progression to NASH. It is now increasingly accepted that bariatric surgery is the most effective method of achieving long-term weight control for patients with morbid obesity[10], with the consequent improvement of the key features of NAFLD and NASH. Thus, weight loss, as a central therapeutic measure, might be combined with antioxidants, in order to minimize or prevent the onset of oxidative stress-induced inflammatory response and insulin resistance, and/or *n-3* LCPUFA, to improve the efficiency of signaling cascades related to

hepatic lipid metabolism and insulin resistance.

Following acute T_3 administration, induction of a mild pro-oxidant state within a time window of 48 h triggers liver preconditioning[54]. This preconditioning strategy has clinical potential, considering that (1) pharmacological and other liver preconditioning maneuvers have not been transferred to clinical applications[53]; and that (2) T_3 is an endobiotic substance, and a widely used and well-tolerated therapeutic agent, which, at low doses, has either no significant or minimal adverse effects that can be readily controlled. However, prevention of IR injury in humans during liver surgery and liver transplantation, using reduced-size grafts from living donors, awaits further experimental and clinical studies.

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ORIGINAL ARTICLE

Aggressive liver resection including major-vessel resection for colorectal liver metastases

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Abstract

AIM: To clarify short- and long-term outcomes of combined resection of liver with major vessels in treating colorectal liver metastases.

METHODS: Clinicopathologic data were evaluated for 312 patients who underwent 371 liver resections for metastases from colorectal cancer. Twenty-five patients who underwent resection and reconstruction of retrohepatic vena cava, major hepatic veins, or hepatic venous confluence during hepatectomies were compared with other patients, who underwent conventional liver resections.

RESULTS: Morbidity was 20% (75/371) and mortality was 0.3% (1/312) in all patients after hepatectomy. Hepatic resection combined with major-vessel resection/reconstruction could be performed with acceptable morbidity (16%) and no mortality. By multivariate analysis, repeat liver resection (relative risk or RR, 5.690; $P = 0.0008$) was independently associated with resection/reconstruction of major vessels during hepatectomy, as were tumor size exceeding 30 mm (RR, 3.338; $P = 0.0292$) and prehepatectomy chemotherapy (RR, 3.485; $P = 0.0083$). When 312 patients who underwent a first liver resection for initial liver metastases were divided into those with conventional resection ($n = 296$) and those with combined resection of liver and major vessels ($n = 16$), overall survival and disease-free rates were significantly poorer in the combined resection group than in the conventional resection group ($P = 0.02$ and $P < 0.01$, respectively). A similar tendency concerning overall survival was observed for conventional resection ($n = 37$) *vs* major-vessel resection combined with liver resection ($n = 7$) performed as a second resection following liver recurrences ($P = 0.09$). Combined major-vessel resection at first hepatectomy (not performed; 0.512; $P = 0.0394$) and histologic major-vessel invasion at a second hepatectomy (negative; 0.057; $P = 0.0005$) were identified as independent factors affecting survival by multivariate analysis.

CONCLUSION: Hepatic resection including major-vessel resection/reconstruction for colorectal liver metastases can be performed with acceptable operative risk. However, such aggressive approaches are beneficial mainly in patients responding to effective prehepatectomy chemotherapy.

Key words: Liver metastases; Colorectal cancer; Liver resection; Major-vessel resection

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INTRODUCTION

Liver resections can be performed with increasing safety for metastatic liver cancer as a result of improved techniques and perioperative care. Major technical complications and fatal liver failure after hepatic resection have become rare. Classically, most reported surgical experience has involved patients with a small number of metastatic lesions in a distribution confined to the hemiliver, but recent advances involving surgical techniques and perioperative care have extended indications for hepatectomy in treatment of colorectal cancer metastases. While extensive hepatectomy, multiple partial liver resections, or both often are necessary to curatively resect aggressive and advanced metastases in the liver, these strategies all involve considerable reduction of hepatic mass, which can lead to clinical decompensation including hepatic insufficiency. Curative resection therefore is not always possible in such patients, despite modern hepatic surgical techniques.

Planned 2-stage hepatectomy, portal vein embolization (PVE), and hepatectomy together with local ablation have been studied as effective ways to completely remove diffuse liver metastases from colorectal cancer[1-4] while preserving functional remnant liver volume and broadening indications for curative resection in these patients. Another strategy is hepatectomy combined with major blood vessel resection and reconstruction. Advanced liver metastases occasionally invade major blood vessels such as the inferior vena cava (IVC), major hepatic veins, or hepatic venous confluence. Complete removal of such tumors requires patients to undergo vascular resection and reconstruction. In the past, involvement of the IVC has been considered a contraindication to resection of advanced liver tumors, because surgical risks were high and long-term prognosis was poor. Presently, liver resection combined with IVC resection and reconstruction has been reported to be a feasible procedure that can be performed with acceptable operative risk and improved long-term outcome in selected patients[5]. However, no definite consensus on long-term survival benefit of such challenging procedures has yet been reached.

In the present study, we retrospectively analyzed patients treated at our institution to estimate efficacy of hepatectomy combined with major blood vessel resection and reconstruction.

MATERIALS AND METHODS

Patients

From April 1992 to March 2009, a total of 394 liver resections for colorectal liver metastases were performed for 334 patients at our Department of Gastroenterological Surgery, Yokohama City University Graduate School of Medicine. A second liver resection was performed in 45 patients with liver recurrence with or without extrahepatic metastases. A third hepatectomy for a second liver recurrence was performed in 11 patients; fourth hepatectomy for third recurrence in 3; and fifth hepatectomy for fourth recurrence in 1. Among the 394 resections, 23 (22 first resections, 1 s resection) were excluded either because curative hepatectomy could not be undertaken or concomitant extrahepatic tumor precluded R0 resection despite curative liver resection. Data from the remaining 312 patients with 371 liver resections were included in the analysis. The mean follow-up duration for these 312 patients after initial liver resection was 49 mo (median, 35; range, 1 to 221). Among these patients, resection and reconstruction of retrohepatic vena cava, major hepatic veins, or hepatic venous confluence was performed during hepatectomy in 25.

Preoperative staging

Preoperative staging included a physical examination, measurement of serum carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9, colonoscopy, barium enema, abdominal ultrasonography, abdominal computed tomography (CT), and chest imaging by routine chest radiography or CT. Imaging by positron-emission tomography was introduced for preoperative staging after 2002.

Hepatectomy procedures

Hepatectomy was not necessarily performed according to anatomic principles of resection; the guiding aim was assurance of tumor-free margins. To determine whether or not a hepatectomy procedure was acceptable for a given patient, we employed a prediction score (PS) introduced by Yamanaka *et al*[6] calculated using the formula; PS = -84.6 + 0.933a + 1.11b + 0.999c. The three variables designated by letter were; a, resection fraction (%) calculated from CT volumetry; b, indocyanine green retention rate at 15 min; c, patient age. A PS less than 50 indicated that a given hepatectomy would be acceptable. When a single-stage combined resection was precluded by insufficient estimated postoperative liver volume, excessive indocyanine green retention rate, or patient age considerations[6] a different strategy was adopted. In such cases PVE, 2-stage hepatectomy, or resection and reconstruction of major vessels during a hepatectomy planned to maximally preserve functional liver parenchyma was performed.

Resection and reconstruction of major vessels were performed as described below. When tumor involvement of the IVC was slight, control of the IVC during resection of the involved portion was achieved simply by placing a vascular clamp in a position tangential to the vena cava; a primary IVC repair then was performed with lateral venorrhaphy, taking care not to narrow the IVC excessively (Figure 1A). Larger resections of the IVC that could not be repaired primarily were reconstructed with synthetic (Figure 1B)[7] or autogenous grafts[8], using venovenous bypass with an active centrifugal force pump if necessary. When the tumor had infiltrated the proximal side of a major hepatic vein or the hepatic venous confluence entering the IVC but extent of tumor involvement of the vein was 2 cm or less,

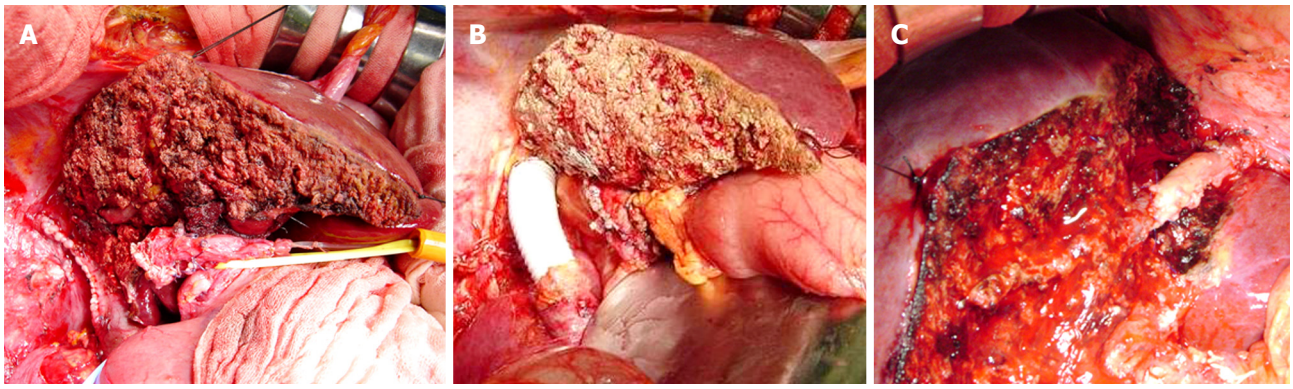


Figure 1 Combined resection of liver and major vessels. A: Extended right hemihepatectomy with resection of the hepatic vena cava repaired primarily; B: Extended right hemihepatectomy with reconstruction of the hepatic vena cava with a Gore-Tex graft; C: Left hemihepatectomy with resection of the middle hepatic venous confluence with the IVC reconstructed using a portal vein graft from the resected liver specimen.

end-to-end anastomosis was carried out. When resection exceeding 2 cm was needed, an autogenous graft of portal vein within resected liver parenchyma was normally used (Figure 1C).

Intraoperative ultrasonography was used to identify any occult tumors not detected preoperatively, and to confirm relationships between tumors and vasculobiliary structures. Parenchymal dissection was performed using ultrasonic dissectors. When necessary, the liver pedicle was clamped intermittently in cycles including 15 min of clamping and 5 min of reperfusion. The Brisbane 2000 terminology of the International Hepato-Pancreato-Biliary Association was used to categorize operative procedures[9].

Any extrahepatic metastases were resected whenever possible, as decided on a case-by-case basis. For resectable metastases in both liver and lung, liver resection and primary tumor resection were performed prior to pulmonary resection, aiming to eliminate the liver as a source of potentially disseminating neoplastic cells. When liver metastases were associated with extrahepatic intra-abdominal metastases, both were resected at the same time.

Principles underlying selection criteria for resection of recurrent hepatic metastases were the same as those for initial hepatectomy. Technical considerations predominated in surgical decisions regarding feasibility of repeat hepatic resection. Since quality and quantity of remaining hepatic parenchyma were highly important factors, patients were excluded from repeat hepatic resection when the PS was greater than 50[6].

Prehepatectomy chemotherapy

Some patients initially deemed to have unresectable liver involvement or patients with marginally resectable metastases (4 or more lesions distributed in 2 lobes; massive tumors; or unfavorably located tumors) underwent prehepatectomy chemotherapy. However, as the choice of treatment depended on several factors including initial assessment of resectability, treatment plans were made on a case-by-case basis. Treatment consisted of infusions into the hepatic artery (HAI) with a combination of 5-fluorouracil (5-FU), l-folinic acid (FA), and cisplatin (CDDP); systemic chemotherapy with 5-FU and FA with or without oxaliplatin or irinotecan; or a combination of both hepatic artery and systemic routes.

Adjuvant therapy

After resection for initial liver metastases, liver recurrence, or extrahepatic recurrence, adjuvant chemotherapy was carried out by HAI or intravenously, generally with 5-FU and FA and with or without addition of CDDP or irinotecan.

Postoperative complications

Among postoperative complications, hyperbilirubinemia was defined as a serum bilirubin concentration on postoperative day 7 of 3 mg/dL or greater. Biliary fistula was diagnosed when bile drainage from the abdominal wound or drain was apparent, with a total bilirubin concentration in the drainage fluid of more than 5 mg/mL or 3 times the serum concentration. Intra-abdominal abscess or liver stump abscess was confirmed by percutaneous drainage. Any medical problems that delayed postoperative recovery and prolonged hospital stay (e.g. ischemic heart disease) also were defined as postoperative morbidity.

Patient follow-up

Patients underwent monthly follow-up evaluation at our outpatient clinic. Data were obtained and recorded from each patient's clinical record. Long-term outcome was ascertained through clinical follow-up, tumor registry follow-up, and contact with the patient, family, or referring physician when necessary. No patients were lost to follow-up. Serum CEA was measured every month, CT was performed every 3 mo, and a chest roentgenogram was obtained every 6 mo for 5 years after the most recent operation.

Statistical analysis

Statistical comparisons of baseline data were performed

Table 1 Outcomes of patients undergoing major vessel resection/reconstruction

Patient No.	Resected vessel (s)	Reconstruction	No. of Hx	Age	Gender	Tumor distribution	No. of tumors	Maximum size (mm)	Resection margin	Outcome month	Status
1	IVC	Primary closure	1	72	F	Bilobar	2	42	Negative	45	DDT
2	IVC	Primary closure	1	70	M	Unilobar	2	15	Negative	67	DDT
3	IVC	Primary closure	1	61	M	Unilobar	1	33	Positive	12	DDT
4	IVC	Primary closure	1	64	M	Bilobar	5	58	Positive	39	DDT
5	IVC	Primary closure	1	70	F	Bilobar	3	75	Negative	33	DDT
6	IVC	Primary closure	1	54	M	Bilobar	27	75	Positive	4	DDT
7	IVC	Primary closure	1	61	F	Bilobar	6	110	Positive	37	DDT
8	IVC	Primary closure	1	72	M	Bilobar	5	20	Negative	11	DDT
9	IVC	Primary closure	1	69	F	Unilobar	1	35	Negative	34	NED
10	IVC	Primary closure	1	71	M	Unilobar	1	55	Positive	31	NED
11	IVC	Primary closure	1	68	F	Unilobar	2	74	Positive	4	NED
12	IVC	Graft replacement	1	70	F	Bilobar	6	63	Negative	16	AWD
13	IVC	Primary closure	2	73	M	Unilobar	1	40	Negative	125	DOD
14	IVC	Primary closure	2	47	F	Bilobar	4	38	Negative	75	DOD
15	IVC	Primary closure	2	65	M	Unilobar	1	42	Positive	38	DDT
16	IVC	Primary closure	2	61	M	Unilobar	1	29	Negative	22	DDT
17	IVC	Patch closure	2	53	F	Bilobar	3	40	Negative	59	DDT
18	IVC	Graft replacement	2	56	M	Unilobar	1	50	Negative	3	NED
19	IVC	Primary closure	3	57	F	Unilobar	1	36	Negative	25	DDT
20	IVC-LHV	Primary closure	3	54	F	Unilobar	1	23	Positive	45	DDT
21	MHV	Graft replacement	1	62	M	Bilobar	4	45	Positive	55	DDT
22	MHV	Graft replacement	1	60	M	Bilobar	2	56	Negative	52	AWD
23	MHV	Graft replacement	2	80	M	Unilobar	1	45	Negative	13	DDT
24	RHV	End-to-end	1	60	F	Unilobar	1	19	Negative	24	DDT
25	RHV	Patch closure	1	55	M	Bilobar	5	17	Positive	71	DDT

Hx: Hepatectomy; IVC: Inferior vena cava; LHV: Left hepatic vein; MHV: Middle hepatic vein; RHV: Right hepatic vein; F: Female; M: Male; N: No; Y: Yes; NED: No evidence of disease; AWD: Alive with disease; DDT: Died of disease treated; DOD: Died of other disease.

by the Mann-Whitney *U* test, the χ^2 test, or Fisher's exact test. Survival rates were calculated by the Kaplan-Meier method. Independent predictors of resection and reconstruction of major vessels being undertaken during hepatectomy were identified by multivariate analysis using multiple logistic regression. Multivariate regression analysis for identifying prognosticators was carried out by a proportional hazard method using a Cox model. Differences between survival curves were analyzed by the log-rank test. A difference was considered significant when the two-sided *P* value was below 0.05.

RESULTS

Details and outcomes in patients with resection/reconstruction of major vessels during hepatectomy

Vascular resection/reconstruction was performed on the IVC alone (n = 19), on the IVC including the confluence of the left hepatic vein (n = 1), on the middle hepatic vein (n = 3), and on the right hepatic vein (n = 2). In the 20 patients with IVC resection, direct suturing of the IVC was performed in 17 patients, an autogenous pericardial patch was applied in 1 patient, and the IVC segment was replaced by a synthetic graft (Gore-Tex; W. L. Gore & Assoc., USA) in 2 patients. All 3 patients with resection of the middle hepatic vein underwent reconstruction of the hepatic vein using a portion of the portal vein within the resected specimen. Vascular continuity was reestablished by end-to-end anastomosis in 1 patient with right hepatic vein resection and by a pericardial patch graft in the other. The patient whose IVC resection/reconstruction used a synthetic graft required venovenous bypass. Patient characteristics and outcomes are shown in Table 1. Negative resection margins were achieved in 15 of these 25 patients. Direct invasion of the IVC wall or major hepatic veins was confirmed histologically in 12 patients. Operative feasibility, hospital stays, and postoperative complications are shown in Table 2. No patients died within 60 d of hepatectomy. Morbidity occurred in 16% (4/25), and 1 patient had both severe ascites and hyperbilirubinemia. Preserved vascular patency was demonstrated by contrast-enhanced CT images approximately 1 mo after resection in all 25 patients with resection/reconstruction of major vessels during hepatectomy. Their 1-, 3-, and 5-year overall survival rates after hepatectomy were 87.0%, 58.6%, and 24.9%, respectively; disease-free rates at these time points were 21.9%, 8.8%, and 8.8%, respectively. mean ± SE and median for survival time in months were 45 ± 7 and 39; mean and median disease-free months respectively were 9 ± 1 and 8 (Figure 2).

Predictive factors of resection/reconstruction of major vessels during hepatectomy

Univariate analysis identified initial *vs* repeat hepatectomy ($P < 0.01$), maximum size of metastases ($P < 0.01$), and prehepatectomy chemotherapy ($P < 0.01$) as significant predictors of resection/reconstruction (Table 3). Multivariate analysis including factors for which univariate

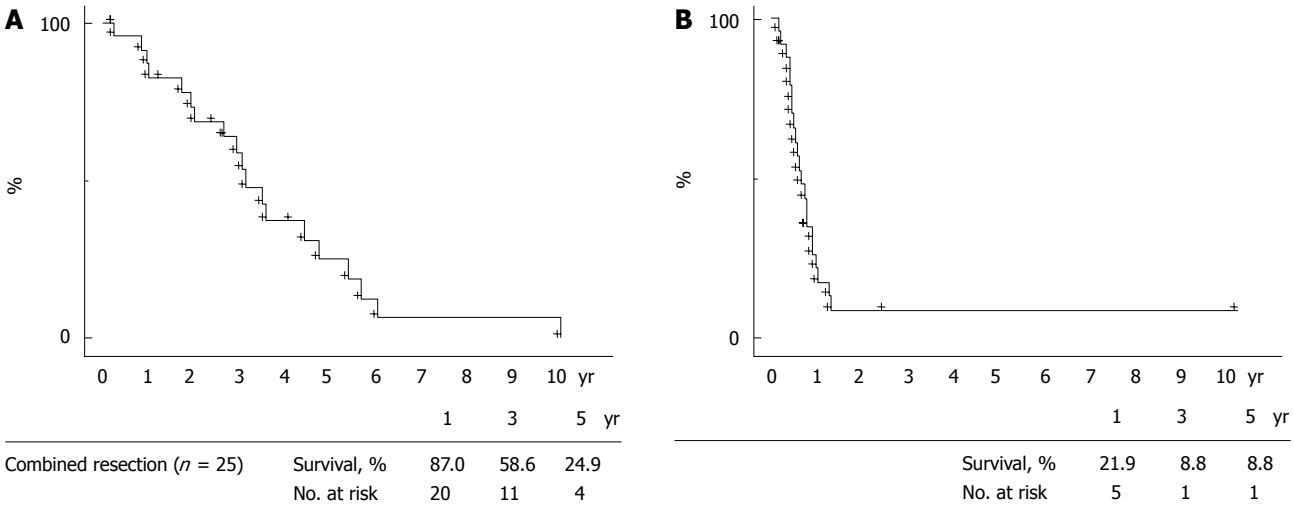


Figure 2 Overall survival (A) and disease-free rate (B) in years following surgery for 25 patients with resection/reconstruction of major vessels during hepatectomy for colorectal liver metastases.

Table 2 Feasibility of combined resection

Variable	Patients (*n* = 25)
Resected liver volume (g)	386 ± 242
(median, range)	(360, 21-972)
Operative time, mean ± SE (min)	488 ± 130
(median, range)	(460, 270-735)
Total blood loss, mean ± SE (L)	1.6 ± 1.3
(median, range)	(1.4, 0.3-5.7)
Patients transfused	17 (68%)
Hospital stay, in days, mean ± SE	19 ± 8
(median, range)	(18, 10-41)
Morbidity	4 (16%)
Ascites	2
Hyperbilirubinemia	1
Bile leakage	1
Intra-abdominal abscess	1

analysis yielded *P* values below 0.1 (initial *vs* repeat hepatectomy, maximum size of metastases, prehepatectomy chemotherapy, primary Dukes stage, extent of hepatectomy, and PVE following hepatectomy) identified 3 factors independently associated with resection/reconstruction of major vessels during hepatectomy: repeat liver resection (relative risk or RR, 5.690; 95% CI, 2.053 to 15.765; $P = 0.0008$), maximum tumor diameter more than 30 mm (RR, 3.338; CI, 1.224 to 9.108; $P = 0.0292$), and prehepatectomy chemotherapy (RR, 3.485; CI, 1.379 to 8.807; $P = 0.0083$; Table 4).

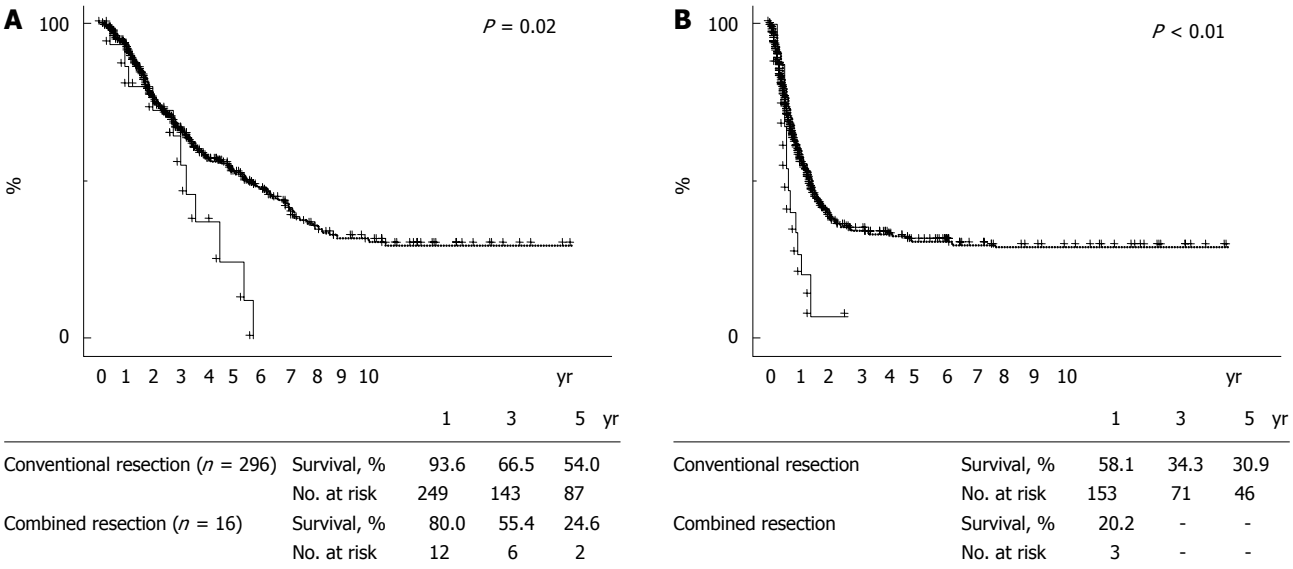
Outcome of hepatectomy with major-vessel resection/reconstruction vs conventional hepatectomy outcome for initial or repeat liver resections

When 312 patients who underwent a first liver resection for initial liver metastases were divided into those with conventional resection ($n = 296$) and those with combined resection of liver and major vessels ($n = 16$), 2 patient- or tumor-related variables, maximum tumor diameter and prehepatectomy CEA, were significantly greater

Table 3 Univariate analysis of predictive factors for combined major-vessel resection

Variables		Conventional (*n* = 346) *n* (%)	Combined (*n* = 25) *n* (%)	*P* value
Patient-related				
Age (yr)	≤ 64	179 (52)	14 (56)	0.84
	≥ 65	167 (48)	11 (44)	
Gender	Male	209 (60)	14 (56)	0.68
	Female	137 (40)	11 (44)	
Primary-related				
Site	Colon	204 (59)	19 (76)	0.14
	Rectum	142 (41)	6 (24)	
Histology	Moderate	223 (64)	14 (56)	0.40
	Others	123 (36)	11 (44)	
Dukes stage	A/B	120 (35)	4 (16)	0.08
	C	226 (65)	21 (84)	
Liver-related				
Hepatectomy	Initial	296 (86)	16 (64)	< 0.01
	Repeat	50 (14)	9 (36)	
Distribution	Unilobar	213 (62)	12 (48)	0.21
	Bilobar	133 (38)	13 (52)	
Number	≤ 2	222 (64)	14 (56)	0.52
	≥ 3	124 (36)	11 (44)	
Maximum tumor size (mm)	≤ 30	197 (57)	6 (24)	< 0.01
	> 30	149 (43)	19 (76)	
Prehepatectomy CEA (ng/mL)	< 10	175 (54)	17 (68)	0.21
	≥ 10	150 (46)	8 (32)	
Treatment-related				
Extent of hepatectomy	Major	112 (32)	13 (52)	0.05
	Minor	234 (68)	12 (48)	
PVE	Performed	44 (13)	7 (28)	0.06
	Not performed	302 (87)	18 (72)	
Staged procedure	Performed	20 (6)	1 (4)	> 0.99
	Not performed	326 (94)	24 (96)	
Hepatectomy with ablation	Performed	26 (8)	4 (16)	0.13
	Not performed	320 (92)	21 (84)	
Prehepatectomy chemotherapy	Performed	63 (18)	11 (44)	< 0.01
	Not performed	283 (82)	14 (56)	

Moderate: Moderately differentiated adenocarcinoma; CEA: Carcinoembryonic antigen; PVE: Portal vein embolization.



		1 yr	3 yr	5 yr
Conventional resection (n = 296)	Survival, %	93.6	66.5	54.0
	No. at risk	249	143	87
Combined resection (n = 16)	Survival, %	80.0	55.4	24.6
	No. at risk	12	6	2

		1 yr	3 yr	5 yr
Conventional resection	Survival, %	58.1	34.3	30.9
	No. at risk	153	71	46
Combined resection	Survival, %	20.2	-	-
	No. at risk	3	-	-

Figure 3 Overall survival (A) and disease-free rate (B) in years since a first liver resection. When patients undergoing initial liver resections were divided into those with combined major vessel resection during hepatectomy (continuous lines, n = 16) *vs* with conventional hepatectomy (broken lines, n = 296), overall survival (panel A, P = 0.02) and disease-free rates (panel B, P < 0.01) were poorer in the combined resection group than in the conventional resection group.

Table 4 Multivariate analysis of predictive factors for combined major vessel resection, by logistic regression analysis

Variables	RR	*P* value
Hepatectomy		
Repeat	5.690 (2.053-15.765)	0.0008
Maximum tumor size (mm)		
> 30	3.338 (1.224-9.108)	0.0186
Prehepatectomy chemotherapy		
Performed	3.485 (1.379-8.807)	0.0083

Values in parentheses are 95% confidence intervals. RR: Risk ratio, followed in parentheses by confidence interval.

in the combined resection group than in the conventional resection group (P = 0.02 and P < 0.01, respectively; Table 5). When survival was compared between these groups, overall survival and disease-free rates were significantly poorer in the combined resection group than in the conventional resection group (P = 0.02 and P < 0.01, respectively, Figure 3). Univariate analysis of these 312 patients identified tumor distribution (P < 0.01), number of metastases (P < 0.01), maximum tumor size (P < 0.01), prehepatectomy CEA (P = 0.01), extrahepatic metastases (P < 0.01), extent of hepatectomy (P < 0.01), tumor-free margin (P < 0.01), PVE (P < 0.01), staged hepatectomy (P < 0.01), prehepatectomy chemotherapy (P = 0.01), adjuvant chemotherapy after resection (P = 0.02), and combined major-vessel resection (P = 0.02) as significant prognostic determinants of the initial resection (Table 6). Multivariate analysis, including factors identified as significant by univariate analysis, identified factors independently affecting survival as number of metastases (≤ 2; RR, 0.543; CI, 0.378 to 0.779; P = 0.0009), prehepatectomy CEA (< 10 ng/mL; RR, 0.683; CI, 0.485 to 0.961; P = 0.0288), extrahepatic metastases (none; RR, 0.549; CI, 0.358 to 0.842; P = 0.0060), staged hepatectomy not performed (RR, 0.481; CI, 0.273 to 0.848; P = 0.0114), use of adjuvant chemotherapy (RR, 0.539; CI, 0.335 to 0.866; P = 0.0107), and no combined major-vessel resection performed (0.512; CI, 0.271 to 0.968; P = 0.0394).

When 44 patients who underwent a second liver resection for liver recurrence were divided into those with conventional resection (n = 37) and those with combined major-vessel resection to liver resection (n = 7), maximum tumor diameter was greater in the combined resection group than in the conventional resection group (P = 0.05, Table 5). Overall survival tended to be poorer in the combined resection group than in the conventional resection group, although significance was not reached (P = 0.09, Figure 4). Univariate analysis identified patient age (P = 0.03), extent of hepatectomy (P = 0.02), adjuvant chemotherapy (P = 0.03), and histologic major-vessel invasion (P < 0.01) as significant prognostic determinants (Table 7). Multivariate analysis identified factors independently affecting survival as extent of hepatectomy (major; RR, 0.264; CI, 0.072 to 0.970; P = 0.0449), use of adjuvant chemotherapy (RR, 0.119; CI, 0.019 to 0.751; P = 0.0235), and lack of histologic major-vessel invasion (0.057; CI, 0.011 to 0.286; P = 0.0005).

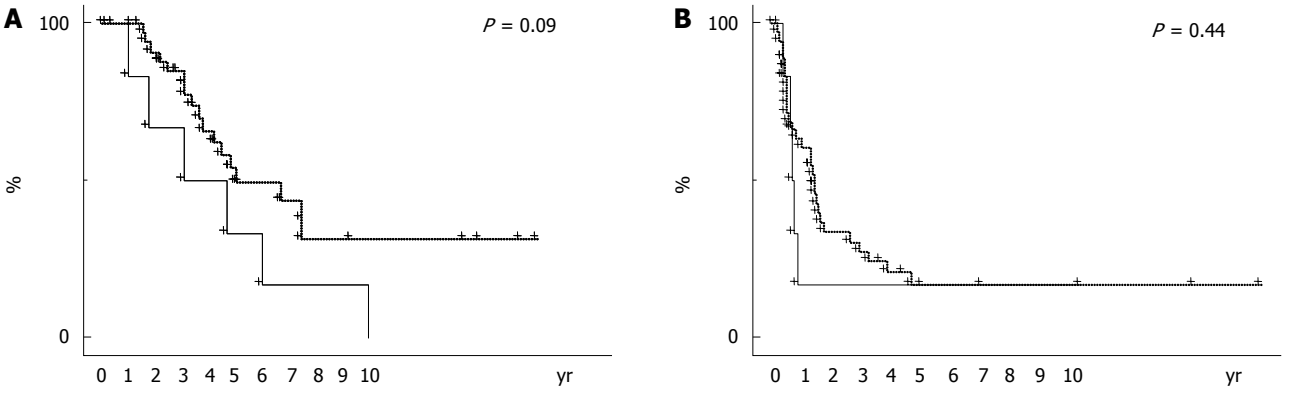
DISCUSSION

In the present study, hepatic resection combined with major blood vessel resection/reconstruction for colorectal liver metastases could be performed with acceptable morbidity and no mortality, although the procedure was associated with greater blood loss and required blood transfusion more frequently than conventional liver resections. For vascular control during combined resections including the IVC, total hepatic vascular exclu-

Table 5 Outcomes of patients undergoing major vessel resection/reconstruction

Variables		Initial resection			Second resection		
		Conventional (*n* = 296)	Combined (*n* = 16)	*P* value	Conventional (*n* = 37)	Combined (*n* = 7)	*P* value
Patient-related							
Age, years		64 (30-85)	66 (54-72)	0.60	63 (32-83)	61 (47-80)	0.91
Gender	Male	186 (63%)	9 (56%)	0.60	19 (51%)	5 (71%)	0.43
	Female	110 (37%)	7 (44%)		18 (49%)	2 (29%)	
Primary-related							
Site	Colon	169 (57%)	11 (69%)	0.61	27 (73%)	6 (86%)	0.66
	Rectum	127 (43%)	5 (31%)		10 (27%)	1 (14%)	
Dukes stage	A or B	103 (35%)	3 (19%)	0.33	13 (35%)	1 (14%)	0.53
	C	193 (65%)	13 (81%)		24 (65%)	6 (86%)	
Histology	Well	99 (33%)	7 (44%)	0.62	11 (30%)	2 (29%)	> 0.99
	Moderate	184 (62%)	8 (50%)		26 (70%)	5 (71%)	
	Others	13 (4%)	1 (6%)		-	-	
Liver-related							
Timing	Synchronous	146 (49%)	10 (63%)	0.44			
	Metachronous	150 (51%)	6 (38%)				
Distribution	Unilobar	175 (59%)	6 (38%)	0.12	27 (73%)	5 (71%)	> 0.99
	Bilobar	121 (41%)	10 (63%)		10 (27%)	2 (29%)	
Number		2 (1-38)	3.5 (1-27)	0.11	1 (1-7)	1 (1-4)	0.57
Maximum tumor size (mm)		28 (5-185)	50 (15-110)	0.02	29 (10-80)	40 (29-50)	0.05
Extrahepatic disease	Present	40 (14%)	4 (25%)	0.26	8 (22%)	1 (14%)	> 0.99
Prehepatectomy CEA (ng/mL)		8.3 (1-10536)	43.9 (2-4498)	< 0.01	8.6 (3-360)	21.5 (2-559)	0.45

Well, well differentiated adenocarcinoma; Moderate, moderately differentiated adenocarcinoma; CEA: Carcinoembryonic antigen.



		1	3	5 yr
Conventional resection (*n* = 37)	Survival, %	100	84.6	53.8
	No. at risk	35	24	12
Combined resection (*n* = 7)	Survival, %	100	66.7	33.3
	No. at risk	6	4	2

		1	3	5 yr
Conventional resection	Survival, %	60.3	27.4	16.7
	No. at risk	21	9	3
Combined resection	Survival, %	16.7	16.7	16.7
	No. at risk	1	1	1

Figure 4 Overall survival (A) and disease-free rate (B) in years since repeat resection for liver recurrence. When patients were divided into a combined resection group (continuous lines, *n* = 7) and a conventional group (broken lines, *n* = 37), overall survival (panel A, *P* = 0.09) tended to be poorer in the combined resection group than in the conventional resection group.

sion[10] and/or hypothermic isolated hepatic perfusion[11] have been used previously. However, most patients in this study did not require venovenous bypass or hypothermic isolated hepatic perfusion, which can involve a clinically significant hemodynamic instability. Such measures could be avoided probably because most of our patients had the IVC reconstructed by primary closure during clamping of a single side of the IVC or total hepatic IVC (clamping below the hepatic venous confluence). The resected IVC can be repaired primarily if

Table 6 Univariate analysis for prognostic factors of the initial resection

Variables		*n*	Survival (%)		*P* value
			3 years	5 years	
Patient-related					
Age (yr)	≤ 64	158	66.9	53.1	0.83
	≥ 65	154	64.9	52.3	
Gender	Male	195	68.2	53.0	0.77
	Female	117	62.4	51.9	
Primary-related					
Site	Colon	180	67.7	53.7	0.87
	Rectum	132	63.5	50.9	
Histology	Moderate	192	67.1	52.1	0.41
	Others	120	64.1	53.5	
Dukes stage	A/B	104	72.2	59.9	0.11
	C	208	62.7	48.8	
Liver-related					
Timing	Synchronous	156	63.7	48.5	0.17
	Metachronous	156	68.1	56.5	
Distribution	Unilobar	181	72.2	59.0	< 0.01
	Bilobar	131	57.2	43.6	
Number	≤ 2	188	72.6	61.2	< 0.01
	≥ 3	124	55.6	39.1	
Maximum tumor size (mm)	≤ 30	169	75.0	61.7	< 0.01
	> 30	143	55.3	41.5	
Prehepatectomy CEA (ng/mL)					
	< 10	158	72.6	60.8	0.01
	≥ 10	145	60.1	44.5	
Extrahepatic metastases	Present	44	40.9	26.7	< 0.01
	Absent	268	70.6	57.3	
Treatment-related					
Extent of hepatectomy	Major	113	57.9	39.8	< 0.01
	Minor	199	70.6	59.8	
Tumor-free margin	Not exposed	256	70.5	58.7	< 0.01
	Exposed	56	46.6	27.9	
PVE	Performed	49	51.0	37.4	< 0.01
	Not performed	263	68.8	55.2	
Staged procedure	Performed	21	33.2	22.1	< 0.01
	Not performed	291	68.5	55.0	
Hepatectomy with ablation	Performed	27	60.5	34.1	0.08
	Not performed	285	66.5	54.2	
Prehepatectomy chemotherapy					
	Performed	68	56.0	35.1	0.01
	Not performed	244	68.4	56.0	
Adjuvant chemotherapy	Performed	257	67.0	54.6	0.02
	Not performed	55	63.1	42.0	
Combined resection	Performed	16	55.4	24.6	0.02
	Not performed	296	66.5	54.0	
Major vessel invasion	Positive	7	66.0	52.9	0.10
	Negative	305	66.0	52.9	

Moderate, moderately differentiated adenocarcinoma; CEA: Carcinoembryonic antigen; PVE: Portal vein embolization.

the resected segment is small[5,12]. Importantly, however, persistent leg edema has been reported when the IVC was narrowed by 50%, despite maintenance of IVC patency[13]. Therefore, the partially resected IVC was often reconstructed using a patch graft. Grafts for patch repair have reportedly included saphenous vein[14], superficial femoral vein[15], and left renal vein[5]. We used a pericardial patch graft for the IVC defect. This graft can be obtained easily even in repeat resections where severe intra-abdominal adhesions may be encountered. This also avoids additional skin incisions and risk of compromising renal function.

Repeat liver resection, large tumors, and prehepatectomy chemotherapy were selected factors predicting resection and reconstruction of major vessels during hepatectomy. A trend associating increased frequency of tumor invasion of major vessels with increased size of metastases readily can be expected. Prehepatectomy chemotherapy was given to patients initially deemed to have unresectable liver involvement or marginally resectable metastases and so one also might expect their tumors to invade major vessels frequently. Distortion and anatomic disorientation caused by rotation of the liver remnant often accompanies regeneration after repeat

Table 7 Univariate analysis for prognostic factors of the second resection

Variables		*n*	Survival (%)		*P* value
			3 years	5 years	
Patient-related					
Age (yr)	≤ 63	23	95.0	62.9	0.03
	≥ 64	21	67.9	34.5	
Gender	Male	24	85.6	56.5	0.21
	Female	20	77.8	43.5	
Primary-related					
Site	Colon	33	82.9	49.1	0.80
	Rectum	11	80.0	54.9	
Histology	Moderate	31	85.9	52.3	0.40
	Others	13	72.7	45.5	
Dukes stage	A/B	14	71.4	38.1	0.15
	C	30	87.9	57.7	
Liver-related					
Distribution	Unilobar	32	79.1	46.5	0.63
	Bilobar	12	90.0	60.0	
Number	1	24	76.1	40.0	0.61
	≥ 2	20	88.9	62.3	
Maximum tumor size (mm)	≤ 30	22	73.3	47.5	0.41
	> 30	22	90.2	52.4	
Prehepatectomy CEA (ng/mL)					
	< 9	18	76.0	53.2	0.56
	≥ 9	18	93.8	67.7	
Extrahepatic metastases	Present	9	77.8	62.2	0.99
	Absent	35	83.2	47.2	
Treatment-related					
Extent of hepatectomy	Major	10	90.0	64.3	0.02
	Minor	34	79.7	46.1	
Tumor-free margin	Not exposed	37	81.2	53.9	0.39
	Exposed	7	85.7	34.3	
Hepatectomy with ablation	Performed	3	66.7	33.3	0.98
	Not performed	41	83.3	52.2	
Prehepatectomy chemotherapy					
	Performed	6	50.0	50.0	0.73
	Not performed	38	80.0	50.0	
Adjuvant chemotherapy	Performed	37	86.1	52.9	0.03
	Not performed	7	0.0	0.0	
Combined resection	Performed	7	66.7	33.3	0.09
	Not performed	37	84.6	53.8	
Major vessel invasion	Positive	4	33.3	0.0	< 0.01
	Negative	40	85.9	54.8	

Moderate, moderately differentiated adenocarcinoma; CEA: Carcinoembryonic antigen.

resections. Repeat resections often induce adhesions of unencapsulated liver surfaces to surrounding organs. Such alteration of anatomy was probably the main reason for repeat resection as a risk factor for major-vessel invasion.

Resection of colorectal liver metastases infiltrating major vessels is technically feasible although its long-term outcome has yet to be fully described. Miyazaki *et al*[5] reported 5-year and median survivals of 22% and 19.2 mo following colorectal metastasis resection combined with IVC resection. Aoki *et al*[16] reported a median survival time for patients with resection/reconstruction of the IVC or hepatic venous confluence of 25.8 mo. Similar results were obtained in the present study; 5-year and median survival of the 25 patients with resection of major vessels were 24.9% and 39 mo after hepatectomy. When patients were divided into conventional resection *vs* combined major vessel resection both at initial and second hepatectomy, overall survival and the disease-free rate in the combined resection group were significantly poorer than in the conventional resection group at initial hepatectomy, although preoperative tumor-related factors (tumor size and CEA) differed significantly between the groups.

Combined major-vessel resection/reconstruction was also identified as a prognosticator at initial hepatectomy for liver metastases by multivariate analysis. Even at a second hepatectomy performed in a limited number of patients with liver recurrence, overall survival tended to be poorer in the combined resection group than in the conventional resection group. As for prognostic factors in the second resection, combined major-vessel resection/reconstruction tended to be a negative prognosticator but fell short of significance by univariate analysis.

The presence of histologic major-vessel invasion was identified as a factor adversely affecting survival. Most

reported surgical experience with combined major-vessel resection/reconstruction for colorectal liver metastases has involved small numbers of patients, precluding definite conclusions about long-term survival. In previous reported series, however, the prognosis for patients with advanced tumors invading the IVC or major hepatic venous confluence seemed unsatisfactory compared to the prognosis for patients without major vessel invasion[5,16]. Impact of combined major-vessel resection/reconstruction on survival may be clearly demonstrated when comparison is made between patients who did not get the surgery and those that did. However, reasons for not performing such surgery were heterogeneous, (intrahepatic and extrahepatic disease status and patients' status), and so it was difficult to obtain similar background characteristics between these patients. Comparison of nutritional or functional assessment was also difficult for the same reasons.

Even in reports including several kinds of liver cancers, 5-year survival was unsatisfactory in cases with vascular invasion, approximately 30%[13,17]. Early tumor recurrence in patients with extensive local tumor spread also has been reported after *ex situ* liver surgery[18]. In treating hepatocellular carcinoma, Yang *et al*[19] reported that portal vein invasion predominated in patients whose first recurrence was in the liver, while hepatic vein invasion was predominant in patients who had only extrahepatic metastases without intrahepatic metastases. When colorectal liver metastases invade the IVC or major hepatic vein, dissemination of tumor cells through these veins may lead to extrahepatic recurrences, as occurs with hepatocellular carcinoma. However, the site of initial recurrence did not differ significantly between our combined and conventional groups after initial liver resection (extrahepatic recurrence, 64% *vs* 64%; $P = 0.82$) or second resection (extrahepatic recurrence, 80% *vs* 54%; $P = 0.07$; data not shown).

Current chemotherapy regimens can achieve either stabilization or decrease in tumor in more than 80% of patients[20,21]. Chemotherapy prior to hepatectomy allows us to extend indications for surgery in the presence of multiple metastases, permitting long-term survival, especially in chemotherapy responders[22-24]. Ng *et al*[25] reported that in response to chemotherapy, death of viable cells is randomly distributed. Necrotic elements in the center of the tumor are replaced by fibrosis, which draws remaining viable cells toward the center, reducing tumor volume. Furthermore, chemotherapy-associated decreases in micrometastases surrounding liver tumors are related to clinical responses and a favorable outcome[22], allowing complete removal of liver tumors to be achieved by a less extensive resection. Therefore, aggressive surgical approaches for liver metastases involving major vessels are best limited to patients showing a response or at least stability during effective prehepatectomy chemotherapy.

Hepatic resection combined with major-vessel resection/reconstruction for colorectal liver metastases can be performed with acceptable operative risk. Although no definite conclusion on long-term survival can be drawn from our study because of a limited number of patients, their overall survival was unsatisfactory. Ongoing advances in perioperative chemotherapy will be necessary to achieve better survival.

COMMENTS

Background

No definite consensus on long-term survival benefit of combined resection of liver with major vessels in treating colorectal liver metastases has yet been reached.

Research frontiers

Only a little information exists about the impact of combined resection of liver with major vessels on the long-term outcome in patients following liver resection for colorectal metastases.

Innovations and breakthroughs

Hepatic resection combined with major-vessel resection/reconstruction for colorectal liver metastases can be performed with acceptable operative risk.

Applications

Surgical approaches for liver metastases involving major vessels are best limited to patients showing a response during prehepatectomy chemotherapy.

Peer review

This study is fair, which can be accepted after answering some questions and revising. In fact, the greatest weakness of this retrospective study is that comparing 25 pts with vascular resection (to create a clear margin without adenoma) to 312 pts without need of vascular resection (to create a clear margin without adenoca) is like the trite old saying "comparing apples to oranges".

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ORIGINAL ARTICLE

Metabolic restaging of hepatocellular carcinoma using whole-body ^{18}F-FDG PET/CT

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Abstract

AIM: To evaluate the ability of ^{18}F-fluorodeoxyglucose positron emission and computed tomography (^{18}F-FDG PET/CT) in restaging of hepatocellular carcinoma (HCC) after treatment.

METHODS: We reviewed a database of the diagnostic performance of ^{18}F-FDG PET/CT scan for patients with HCC following local or regional treatment. The database consisted of ^{18}F-FDG PET/CT information of 21 male and 4 female (age range, 27-81 years; mean age, 51.6 years) patients who had received surgical resection and/or interventional treatments and then underwent ^{18}F-FDG PET/CT scan. All patients had received enhanced CT scan of the liver two weeks before or after the ^{18}F-FDG PET/CT scan. Intrahepatic recurrence and/or extrahepatic metastases were confirmed by histological analysis or clinical and imaging follow-up. The accuracy of ^{18}F-FDG PET/CT study was determined by histopathological results or by clinical and imaging follow-up.

RESULTS: ^{18}F-FDG PET/CT was abnormal in 19 of the 25 (76.0%) patients. In detecting HCC recurrence, ^{18}F-FDG PET/CT scored 17 true positives, 5 true negatives, 2 false positives and 1 false negative. The sensitivity, specificity and accuracy of ^{18}F-FDG PET/CT in detecting HCC recurrence was 89.5%, 83.3% and 88%, respectively. ^{18}F-FDG PET/CT had an impact on management of these patients by settling the problem of an unexplained increase in alpha-fetoprotein after treatment (14 patients), by monitoring response to the treatment and guiding additional regional therapy (12 patients), by identifying extrahepatic metastases (10 patients), by identifying tumor growth or thrombosis in the portal vein (6 patients), or by guiding surgical resection of extrahepatic metastases (2 patients).

CONCLUSION: Our results suggest that whole body ^{18}F-FDG PET/CT may be useful in the early evaluation of residual, intrahepatic recurrent or extrahepatic metastatic lesions and able to provide valuable information for the management of HCC recurrence.

Key words: ^{18}F-fluorodeoxyglucose; Positron emission tomography/computed tomography; Hepatocellular carcinoma; Surgeon resection; Interventional treatment; Residual lesion; Intrahepatic recurrence; Extrahepatic metastases; Restaging

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Sun L, Guan YS, Pan WM, Luo ZM, Wei JH, Zhao L, Wu H. Metabolic restaging of hepatocellular carcinoma using whole-body ^{18}F-FDG PET/CT. *World J Hepatol* 2009; 1(1): 90-97 Available from: URL: http://www.wjgnet.com/1948-5182/full/v1/i1/90.htm DOI: http://dx.doi.org/10.4254/wjh.v1.i1.90

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common solid malignancies worldwide, with up to 1 million new cases per year. Its mortality is second to lung cancer in urban and to gastric carcinoma in rural regions of China[1,2]. Surgical treatments, including hepatic resection and liver transplantation, are considered as the most effective treatment of HCC. However, less than 20% of HCC patients can be treated surgically[3]. Interventional treatments have been applied to patients with inoperable HCC[4,5]. Despite initial remission of HCC, after surgical and interventional treatments, recurrence is common. Since patients with recurrent HCC may be amenable to potentially curative resection, early detection of intrahepatic recurrence and/or extrahepatic metastases is extremely important and can facilitate successful retreatment at an early stage. Late diagnosis makes retreatment difficult[6,7].

Conventional computed tomography (CT) and magnetic resonance imaging (MRI) are the main techniques that are used during the follow-up of HCC. However, these techniques may not be reliable enough in detecting residual, recurrent or metastatic lesions[8,9]. The reported increase in sensitivity of ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT) over CT and magnetic resonance imaging (MRI) has been attributed to the ability of ^{18}F-FDG PET/CT to detect metabolic abnormalities that precede the morphological changes seen by CT[10]. This study was undertaken to further define the usefulness of ^{18}F-FDG PET/CT imaging in evaluating residual, intrahepatic recurrent or extrahepatic metastatic lesions of HCC after primary treatment.

MATERIALS AND METHODS

Patients

During the period from January 2007 to Oct July 2008, thirty ^{18}F-FDG PET/CT scans were performed in 25 patients (age range, 27-81; 21 men and 4 women), who had undergone both/either surgical resection and/or interventional therapy for HCC. These patients, with/without suspicious intrahepatic recurrence and/or extrahepatic metastases in conventional imaging or according to clinical findings, were retrospectively enrolled in our study. All patients had received enhanced CT scan of the liver during the two weeks preceding or following the ^{18}F-FDG PET/CT scan. The standard for the ultimate diagnosis of tumor recurrence consisted of histopathological confirmation or of clinical and imaging follow-up information for at least 12 mo after the PET/CT examination.

^{18}F-FDG PET/CT technique

Patients were asked to fast for at least 4 h before undergoing ^{18}F-FDG PET/CT examination. Their blood glucose level had to be within the normal range (70-120 mg/dL) prior to the intravenous injection of ^{18}F-FDG with a radiation dosage within 370 and 666 MBq (10-18 mCi). Data acquisition was performed within 60 min after the injection with an integrated PET/CT system (Discovery STE; GE Medical Systems, Milwaukee, WI, USA). The procedure of data acquisition was as follows: the CT scan was performed covering the patient from the head to the pelvic floor, with 110 kV, 110 mA, tube rotation time of 0.5 s, and 3.3-mm section thickness, which was matched to the PET section thickness. Immediately after CT scanning, a PET emission scan, covering the same transverse field of CT view, was obtained. Acquisition time was 3 min per table position. PET image data sets were reconstructed iteratively by applying the CT data for attenuation correction. Finally, coregistered images were displayed on a workstation.

PET/CT image interpretation

Reviewer 1 and reviewer 2, who were aware of the clinical and other imaging data, read the ^{18}F-FDG PET/CT images on a high-resolution computer screen. The reviewers reached a consensus in cases of discrepancy. Reviewer 1 had 21 years of experience in both nuclear medicine and radiology, and reviewer 2 had 6 years of experience in both expertises. If a focus within the liver had hypermetabolic activity greater than that of the adjacent normal liver tissue, it was considered intrahepatic recurrence. ^{18}F-FDG PET/CT scan was considered positive or suspiciously positive for metastases whenever abnormal non-physiologic metabolic activity was identified at extrahepatic sites. Diffuse mild activity in the intestinal tract was considered normal physiologic uptake. Quantification of tumor metabolic activity was obtained using the Standardized Uptake Value (SUV) normalized to body weight. mean ± SD of maximum-pixel SUV (SUVmax) of the lesions were calculated.

RESULTS

Clinical presentation of recurrent disease

The characteristics of the patients are shown in Table 1. Mean time after treatment to PET/CT exam was 13 mo (10 d-12 year) and mean follow up time after PET/CT exam was 12 mo. At the time of intrahepatic recurrence and extrahepatic metastases being suspected, the mean patients' age was 51.6 years with a tendency to a preponderant male gender distribution (84%). The suspicion of intrahepatic recurrence and/or extrahepatic metastases leading to a ^{18}F-FDG PET/CT was based on an unexplained increase in alpha-fetoprotein (AFP) (n = 14) (Figure 1), suspected intrahepatic recurrence routine follow-up after TACE (n = 8), or suspected extrahepatic metastases at CT or MRI (n = 7). The features of recurrent HCC after treatment are summarized in Table 2.

Residual, intrahepatic recurrent or extrahepatic metastatic lesions

The accuracy of ^{18}F-FDG PET/CT detection was determined by histopathological examination or based on other clinical evidence. The ^{18}F-FDG PET/CT of the

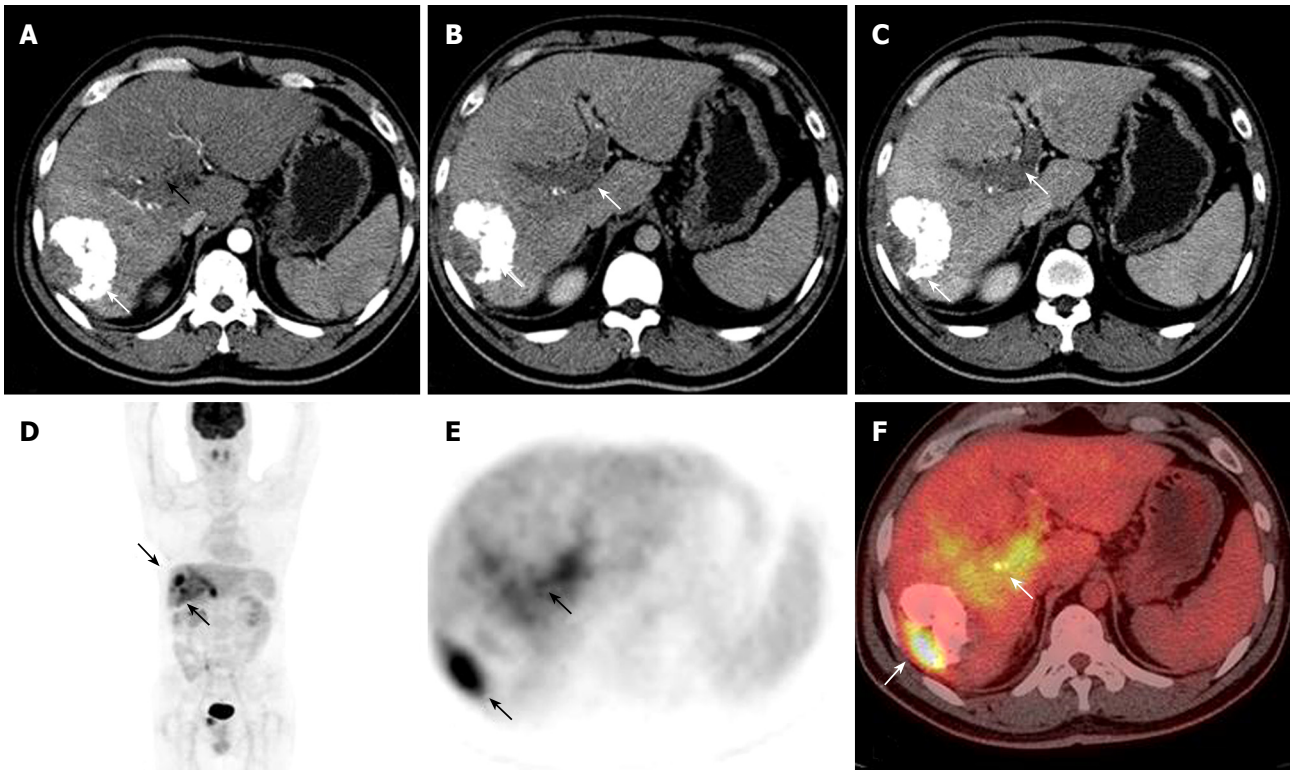

Figure 1 A 38-year man, who had TACE 1 mo before, underwent PET/CT to monitor response to the treatment. Contrast-enhanced arterial-phase axial CT image showed the mass with partial accumulation of iodized oil in the right lobe of the liver (arrows, A, B). A filling defect was detected in the left branch during the portal phases (arrow, C). PET and PET/CT fused images (arrows, D, E and F) revealed residual viable tumor and a highly metabolically active tumor thrombus in the left branch of the portal vein.

Table 1 Patients' features

Clinical characteristics	Data
Mean age (yr)	51.6 (range 27-81 yr)
Gender	
Males	21
Females	4
Prior therapy	
TACE	6
Liver resection	9
TACE after surgery	8
TACE + RFA or PEI	2
Unexplained AFP increase after treatment	14
Mean time from treatment to recurrence	11.5 mo
Mean time after treatment to PET/CT exam	10 d - 12 yr; mean 13 mo
Mean follow up time after PET/CT exam	3 mo - 21 mo; mean 12 mo

Table 2 Characteristics of recurrent HCC in 17 true positive patients

Characteristics	*n*
Intrahepatic recurrence	8
Intrahepatic recurrence and extrahepatic metastases	5
Extrahepatic metastases without intrahepatic recurrence	4
Single intrahepatic recurrence	7
Multiple intrahepatic recurrence	8
Lung	4
Bone	1
Omentum and mesentery	3
Retroperitoneal lymph nodes	2
Adrenal	1

25 cases of HCC with a post-treatment follow-up lead to 17 true positive (Figures 2 and 3), 1 false negative, 5 true negative and 2 false positive results. The 2 false positive PET/CT findings were later verified as post-operative inflammation (Figure 4). Regarding the single patient with a false negative PET/CT, the final diagnosis was low-level metabolism metastasis of the left adrenal gland.

The sensitivity, specificity and accuracy levels of the ^{18}F-FDG PET/CT in detecting post-treatment HCC recurrence were 89.5%, 83.3% and 88%, respectively. ^{18}F-FDG PET/CT imaging had an impact on management of these patients, by solving the problem of an unexplained increase in AFP after treatment (14 patients), by monitoring the response to treatment and guiding additional regional therapy (12 patients), by identifying extrahepatic metastases (10 patients), by identifying tumor growth or thrombosis in the portal vein (6 patients), or by guiding surgical resection of extrahepatic metastases (2 patients).

Of the cases with positive post-treatment findings, 90% occurred within the first 1 year, while less than 9% occurred after 2 years. Positive findings were frequently detected as both intrahepatic lesions and extrahepatic metastases.

Afp and recurrent disease

^{18}F-FDG PET/CT study was performed in 14 patients,

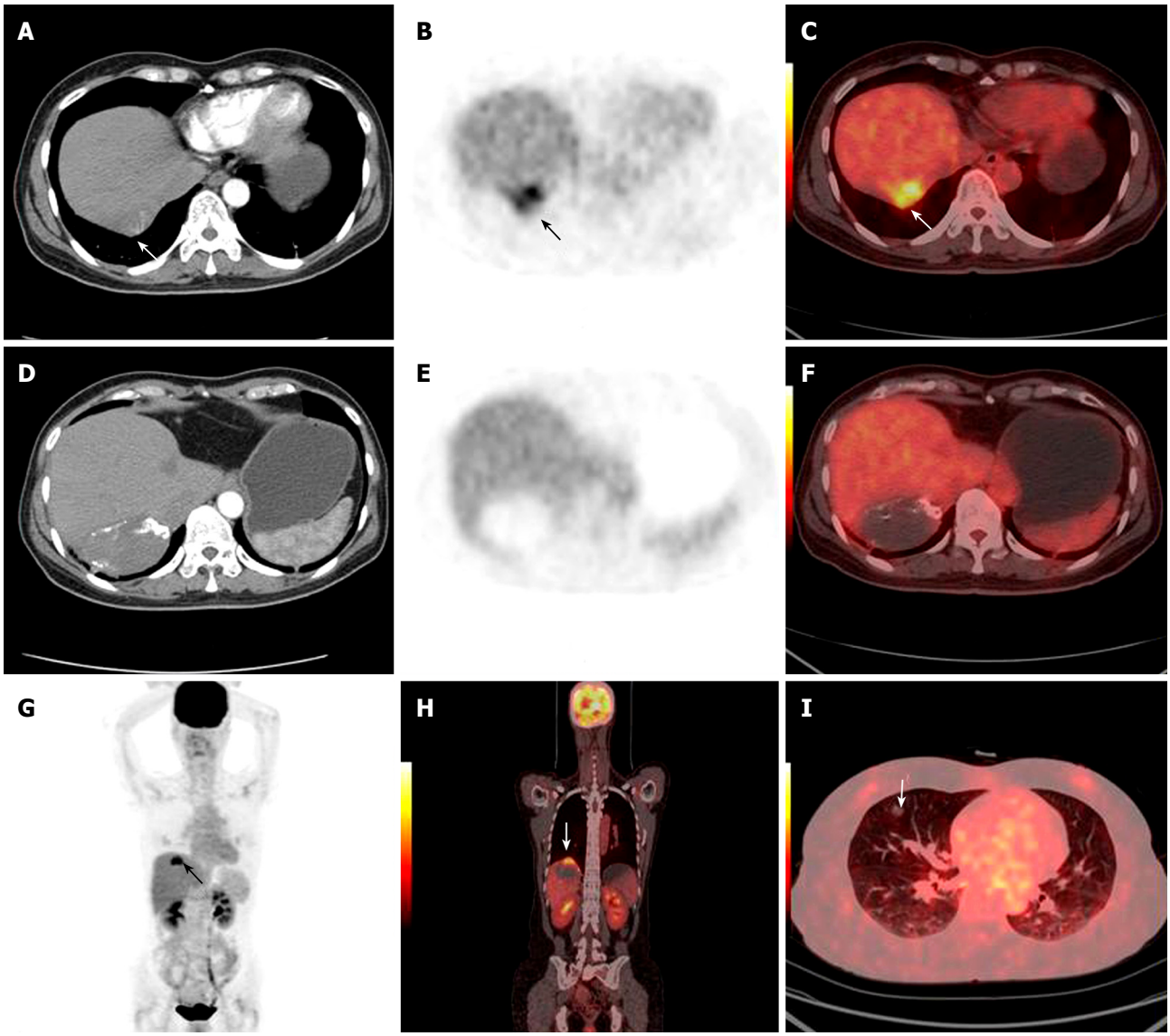

Figure 2 A 50-year woman who had HCC resection 2 years before had an intrahepatic HCC recurrence and received combined RFA and TACE treatment. A highly metabolically active lesion was detected on the top of the lesion by PET and PET/CT fused images (white arrows, A-C and G). Contrast-enhanced CT image showed the non enhanced mass with faint accumulation of iodized oil in the right lobe of the liver (D-F). PET/CT fused images revealed a low FDG uptake benign lesion in the right lung (I). All findings were later verified by clinical follow-up.

who had undergone either surgical resection or interventional therapy for HCC, but who had subsequently developed increasing serum levels of AFP during routine follow-up. In 5 of these patients, ^{18}F-FDG PET/CT was carried out due to suspected disease recurrence upon liver enhanced CT scan. The remaining 9 had high post-treatment AFP serum levels during routine follow-up, but liver enhanced CT scan and physical examinations were normal. ^{18}F-FDG PET/CT findings were abnormal in 11 of the 14 patients with unexplained increase in AFP after treatment. Intrahepatic lesions were detected in 8 and extrahepatic metastases were found in 3 patients with abnormal PET/CT findings.

DISCUSSION

Mortality due to HCC ranks as the second highest in cancer-related deaths nationwide and its morbidity is increasing among the male population[11]. In the past decades, numerous non-surgical interventional therapies have been continuously introduced as a result of the technical developments of locoregional approaches for HCC. The therapy consisting in surgical resection combined with interventional treatment has played an important role in the treatment of HCC[12]. However, intrahepatic recurrence and extrahepatic metastases are still the major obstacles to improve further the prognosis of HCC. The recurrence rate of intrahepatic carcinomas varies from 36.8% to 82%, while 30% of the recurrences are extrahepatic[13]. The 5-year recurrence rates are, respectively, 38% and 61.5%, while the 5-year disease-free survival are 16% or 38.6% after curative resection; overall, recurrent HCC is the single most important cause of death after treatment[14].

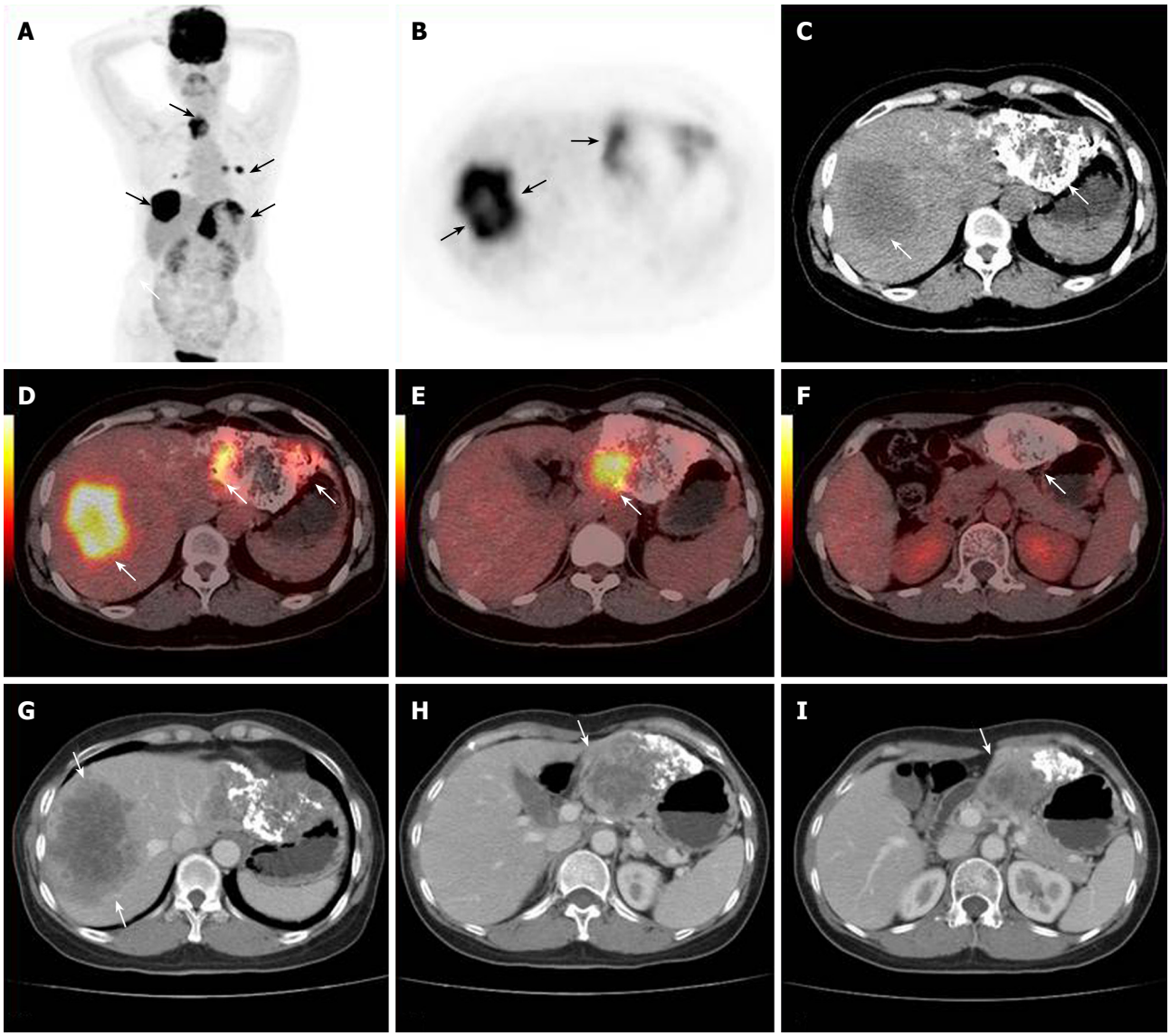

Figure 3 A 55-year woman suffering from HCC received TACE treatment. Extrahepatic metastases (arrows, A) were detected by PET and PET/CT fused images. Highly metabolic new recurrent lesion of the right lobe (arrows, B-D) and a resident lesion of the left lobe (arrows, E, F) were detected by PET/CT fused imaging. Contrast-enhanced CT follow-up after 1 mo later confirmed the progression of lesions.

Despite the high recurrence rate of HCC, long-term survival can be achieved after treatment by early detection. This is critical, because recurrences confined to the liver may be amenable to treatment with an additional survival benefit. Surgical resection for isolated extrahepatic recurrence of HCC is also recommended to increase long-term survival[15,16]. So far, contrast enhanced CT or MRI are considered as the most sensitive test for assessing therapeutic efficacy. These imaging examinations play a crucial role in the follow-up of HCC treated by surgical resection and interventional procedures, since local treatment efficacy, recurrent disease and some of therapy-induced complications can be evaluated[17,18]. However, CT and MRI have both advantages and disadvantages in the evaluation of local treatment efficacy, recurrent disease and some of the therapy-induced complications, while local CT or MRI have also limitations in the detection of extrahepatic HCC[19,20].

Major advantages of whole body ^{18}F-FDG PET/CT are the capability to perform full-body examinations, the potential to detect intrahepatic recurrence and extrahepatic metastases in one single examination and the possibility of distinguishing new active disease from scar or necrotic tissue[21]. The whole-body nature of the ^{18}F-FDG PET/CT study also contributes to the increased sensitivity through detection of distant metastatic lesions[22]. Tumors with increased ^{18}F-FDG uptake are more metabolically active and biologically aggressive. The degree of ^{18}F-FDG uptake and its distribution within the tumor and the surrounding hepatic parenchyma could provide useful information for specifying the degree of tumor necrosis and working out strategies for subsequent additional locoregional therapy[23,24].

AFP is a useful marker for monitoring treatment effects on HCC patients. It is considered a relatively specific test for HCC among high-risk patients, particu-

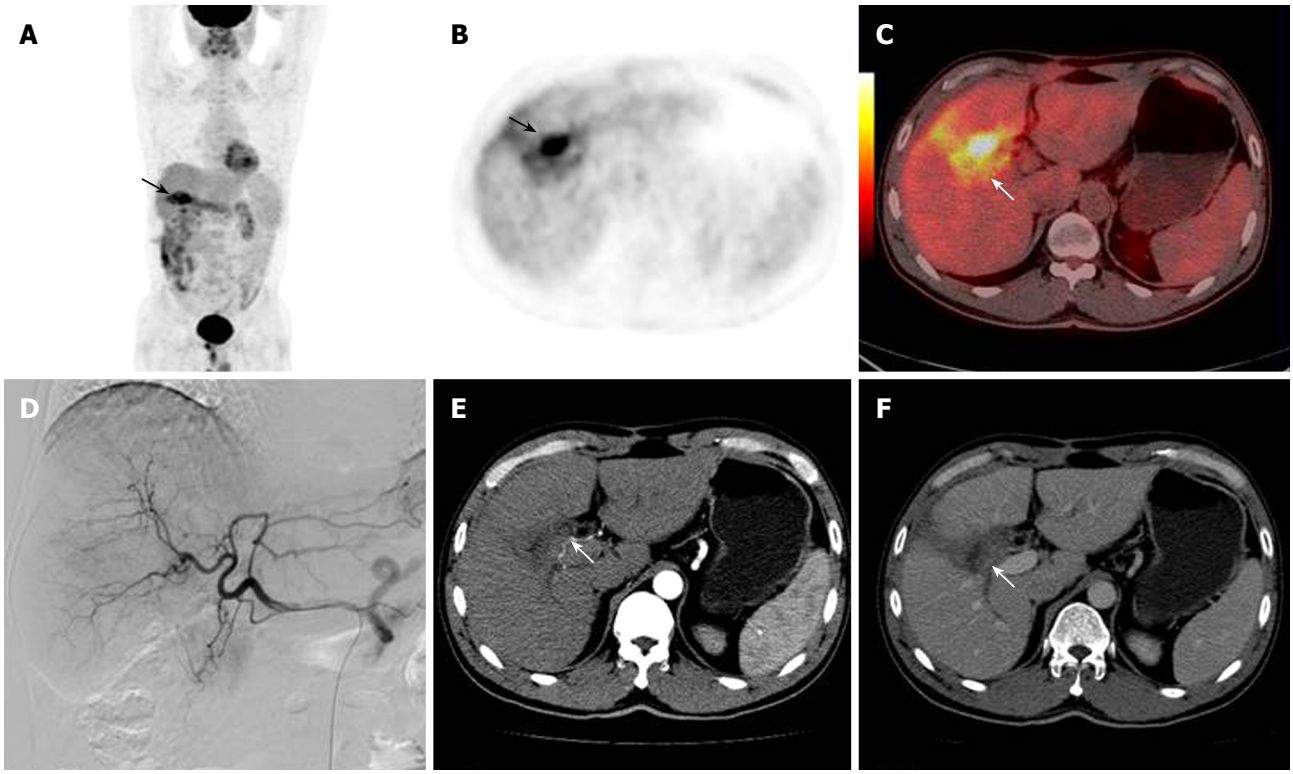

Figure 4 A 48-year man who had HCC resection 2 mo before the imaging, followed by TACE 40 d later. PET and PET/CT fused images revealed highly metabolic lesions (arrows, A-C). Both contrast-enhanced axial CT imaging and arteriography failed to show lesions in the right lobe of the liver (arrows, D-F). These findings of PET/CT were later verified as inflammatory by post-operative tissue examination.

larly those with advanced disease and with levels tending to increase in follow-up examinations. However, measurements of AFP have been demonstrated to have limited value for detecting recurrence at an early stage. In addition, elevated AFP level may also be transiently present in patients with cirrhosis and/or hepatitis[25,26]. In some patients with elevated serum level of AFP, no tumors can be detected by conventional enhanced CT and MRI scanning. Our results suggest that ^{18}F-FDG PET/CT is a valuable imaging tool in patients who have rising AFP levels after HCC treatment, even when conventional examinations are normal. Whole-body ^{18}F-FDG PET/CT scan provides also an important and valuable imaging study for detecting extrahepatic metastases.

Iodized oil is widely used in transcatheter arterial chemoembolization (TACE). It is difficult to completely kill the tumor cells with TACE in only one session, and it is very important to objectively assess the viability and necrosis of the tumors after TACE, in order to assess the indication to additional treatment sessions, with the aim to improve the general therapeutic effects and the survival rate[27]. Generally, the CT follow-up of patients treated with TACE alone could be affected by artifacts produced by high local concentrations of lipiodol, making it difficult to evaluate the characteristics of the lesion. On the other hand, the homogeneous and complete deposition of lipiodol within the lesion would indicate a high degree of necrosis of the tumors: however, it is difficult to estimate correctly the viability and necrosis of the tumors in case of inhomogeneous deposition, because lipiodol-negative areas do not correspond to viable areas of the tumors[28,29]. On the contrary, whole-body ^{18}F-FDG PET/CT scan may represent an important and valuable imaging tool to detect residual, intrahepatic metastatic or extrahepatic metastatic lesions. In particular, the capability of detecting residual lesions is not affected by high concentrations of lipiodol.

The same diagnostic criteria described above for lesions treated with TACE can be applied to assess the therapeutic responses to percutaneous ethanol injection (PEI) and radio-frequency ablation (RFA). The imaging appearances of the lesions after these two treatments are very similar. However, the absence of contrast enhancement within the ablated lesion at CT and MRI performed during follow-up after treatment cannot always indicate a successful treatment, as later follow-up studies may demonstrate tumor regrowth at the periphery of the ablated lesion[30]. ^{18}F-FDG PET/CT is useful to assess residual HCC after treatment with RFA and to guide its further local treatment.

HCC carries a high risk of invasion of the portal vein. The detection and etiologic characterization of these thrombi are essential for treatment planning, particularly in patients with hepatic tumors, because malignant thrombosis is a negative prognostic factor. The management of HCC with portal vein tumor thrombosis is complicated and controversial. In our group, five consecutive patients with biopsy-proven HCC, and thrombosis of the main portal vein and/or left/right portal vein on ultrasound (US), CT or MRI were identified

by ^{18}F-FDG PET/CT. ^{18}F-FDG PET/CT showed a highly metabolically active thrombus in 4 of the 5 patients. ^{18}F-FDG PET/CT scored a true positive result in all 4 patients, and a true negative result in the other patient. No false positive results were observed using ^{18}F-FDG PET/CT[31,32].

Resection of isolated extrahepatic HCC metastases has been advocated to obtain long-term survival. Accurate re-staging plays the most important role in making a decision on extrahepatic metastases resection. In our study, in a 60-year old woman with increasing AFP levels after HCC resection, conventional chest and abdominal CT did not find any evidence of intrahepatic recurrence or extrahepatic metastases. ^{18}F-FDG PET/CT detected a lymph node with high metabolic activity at the level of the omentum. After excision of this lymph node, histopathological examination revealed a metastatic HCC and AFP level returned to normal. This case shows that ^{18}F-FDG PET/CT may provide a highly significant, additional information regarding the accurate detection of extrahepatic spread of tumors and additional important information in patients with presumably resectable extrahepatic metastases[33]. This test should be used for patients at high risk of extrahepatic disease and should be evaluated prospectively in all patients considered for resection of intrahepatic recurrence and extrahepatic metastases[34].

The diagnostic contrast-enhanced multiphase CT, as part of the combined ^{18}F-FDG PET/CT protocol, has the potential to provide considerable added value in specific clinical conditions leading, as a result, to a change in the management of a substantial proportion of patients. The greatest benefit of the diagnostic CT lies in the possibility of localizing a pathological FDG uptake and of correctly delineating the tumor boundaries, leading to changes in the interpretation of ^{18}F-FDG PET/CT, at least in some patients.

Our study has some limitations. First, it was a retrospective analysis. Second, we recognize that not all of the extrahepatic lesions had evidence for metastatic HCC at biopsy. However, in a patient with a known HCC and with no other primary tumor, the development of a new lesion (e.g. a new bone lesion) or the increase over time of previously diagnosed extrahepatic lesions strongly suggest metastatic HCC. These criteria for metastatic disease are used by oncologists and surgeons in planning therapy.

In conclusion, High recurrence rates after treatment of HCC indicate that there is substantial room for improvement of current imaging strategies for the early detection of recurrent lesions. Whole body ^{18}F-FDG PET/CT scan may provide valuable information for the early detection and guide salvage treatment for recurrent HCC.

COMMENTS

Background

Despite initial remission of hepatocellular carcinoma (HCC) after surgical and interventional treatments, recurrence is common. Since patients with recurrent HCC may be amenable to potentially curative resection, early detection of intrahepatic recurrence and/or extrahepatic metastases is extremely important and can facilitate successful retreatment at an early stage. Late diagnosis makes retreatment difficult. This study was undertaken to further define the usefulness of ^{18}F-fluorodeoxyglucose positron emission and computed tomography (^{18}F-FDG PET/CT) imaging in evaluating residual, intrahepatic recurrent or extrahepatic metastatic lesions of HCC after primary treatment.

Research frontiers

^{18}F-FDG PET and, particularly, ^{18}F-FDG PET/CT are widely accepted imaging methods in the management of a wide variety of cancers. The reported increase in sensitivity of ^{18}F-FDG PET/CT over CT and MRI has been attributed to the ability of ^{18}F-FDG PET/CT to detect metabolic abnormalities that precede the morphologic changes seen by CT. However, the usefulness and limitations of ^{18}F-FDG PET/CT in evaluating residual, intrahepatic recurrent or extrahepatic metastatic lesions of HCC after primary treatment still need further clinical evaluation.

Innovations and breakthroughs

Whole body ^{18}F-FDG PET/CT was effective in detecting relapse in evaluating residual, intrahepatic recurrent or extrahepatic metastatic lesions of HCC after primary treatment and also had important clinical impacts on the management of recurrent HCC.

Applications

High recurrence rates after treatment of HCC indicate that there is substantial room for improvement of current imaging strategies for the early detection of recurrent lesions. Whole body ^{18}F-FDG PET/CT scan could provide valuable information for early detection and might guide salvage treatment for recurrent HCC.

Peer review

This is an interesting report of PET and PET/CT for HCC. Whereas it is of clinical significance, the presentation of the paper needs some modification. It deserves some comments for further consideration.

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BRIEF ARTICLE

Partial blockage of hepatocyte maturation in hepatoma-derived growth factor transgenic mice

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Abstract

AIM: To investigate the role of hepatoma-derived growth factor (HDGF) in liver development, especially in the hepatocyte differentiation.

METHODS: We generated transgenic mice which overexpressed HDGF in hepatocytes under the transcriptional control of mouse albumin promoter/enhancer. To examine the effects of HDGF overexpression on hepatocyte differentiation, we investigated the expression patterns of the differentiation marker genes.

RESULTS: The HDGF transgenic mice developed normally and showed no apparent abnormality in the liver. However, the gene expression patterns of the liver in adult transgenic mice were similar to those of the neonatal liver in control mice.

CONCLUSION: These findings suggest that HDGF-overexpression partially suppresses hepatocyte maturation.

Key words: Hepatoma-derived growth factor; Hepatocyte; Maturation; Transgenic mice

Peer reviewer: Nathalie Wong, Professor, Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Shatin NT, Hong Kong, China

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INTRODUCTION

The liver is the major hematopoietic organ during the fetal period, and immature hepatocytes function as stromal cells which support hematopoiesis. During liver development, immature hepatocytes differentiate and acquire many functions in preparation for the metabolic conditions after birth[1,2]. The expression patterns of differentiation marker genes can represent the maturational stage of hepatocytes. Alpha-fetoprotein (AFP) is one of the early marker genes of the immature hepatocytes and its expression remarkably decreases after birth[3]. The expression of albumin, the most abundant protein synthesized by hepatocytes, begins during the mid-gestational stage, and this expression increases with the progression of liver development, especially after birth[4]. In the late-gestational stage hepatocytes begin to produce metabolic enzymes including tyrosine amino transferase (TAT) and glucose-6-phosphatase (G-6-Pase)[5,6]. Subsequently, hepatocytes

gain a fully matured phenotype, characterized by the expression of tryptophan oxygenase (TO) within two weeks after birth[7]. The expression levels of TAT and G-6-Pase peak in the neonatal liver and decrease in the adult liver. In contrast, TO is barely expressed in the fetal and neonatal liver and is highly expressed in the adult liver. Although the gene expression patterns of hepatocytes continue to alter after birth, few studies have documented the growth and differentiation of post-natal hepatocytes.

Hepatoma- derived growth factor (HDGF) is a heparin-binding protein, which has been identified from the conditioned media of HuH-7 hepatoma cells[8,9]. HDGF stimulates the proliferation of fibroblasts, endothelial cells, vascular smooth muscle cells and hepatocytes[8-11]. Its primary sequence contains nuclear localization signals and the HDGF can be transported to the nucleus, thus indicating that HDGF is a unique nuclear/growth factor[12,13]. Recently, several novel genes have been identified for proteins which share a highly homologous amino terminal region consisting of about 100 amino acids; so-called HDGF- related proteins (HRPs)[14,15]. It is thought that HDGF and HRPs form a new gene family. Although HDGF was initially identified in human hepatoma-derived cells, HDGF mRNA is expressed in various normal adult tissues of mice and humans, thus suggesting that HDGF has some physiological functions in non-tumor cells[9].

Previous studies have suggested that HDGF participates in fetal organ development and adult tissue repair as an autocrine growth factor[10,11,16]. We have shown that HDGF is highly expressed in immature fetal hepatocytes and promotes their proliferation[16]. Furthermore, HDGF is induced in two animal models of liver regeneration[17], suggesting that HDGF plays an important role in the proliferation of both fetal and adult hepatocytes. Although the involvement of HDGF in cell differentiation has not been clarified, the suppressive effects of HDGF on gut cell maturation have been suggested[18]. We generated transgenic mice which overexpressed HDGF in hepatocytes under the control of the albumin promoter/enhancer, in order to examine the functional role of HDGF in liver development. The gene expression patterns of hepatocytes in adult transgenic mice resemble those of neonatal hepatocytes of wild-type mice, thus suggesting that HDGF-overexpression partially suppresses hepatocyte maturation.

MATERIALS AND METHODS

Mice

The DNA fragment covering the entire coding region of mouse HDGF was cloned into the Eco RI site of an expression vector which contains the promoter and enhancer of the mouse albumin gene[19]. A schematic representation of the constructed transgene (Alb-HDGF) is illustrated in Figure 1. Transgenic founders were generated by pronuclear injection according to standard techniques. Using a ^{32}P-labeled fragment of HDGF-specific cDNA as a probe, transgene integration and expression were identified by Southern and Northern blot analyses, respectively. C57BL/6CrSlc mice (Nihon SLC, Shizuoka, Japan) or non-transgenic mice were used as controls. All animal experiments were performed according to the guidelines of Osaka University Medical School.

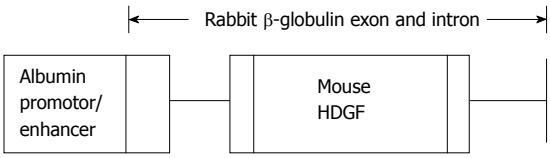

Figure 1 Schematic representation of the constructed transgene of HDGF. Schematic representation of the constructed fragments used in the generation of transgenic mice. A DNA fragment covering the entire cDNA of the mouse HDGF was inserted into the expression vector, which contains promoter and enhancer of mouse albumin gene.

Hybridization probes

The probes used for the Northern blot analysis were as follows: a 0.4 kb fragment of mouse HDGF cDNA[9], a 0.5 kb fragment of mouse G-6-Pase cDNA[20], and TO cDNA[20].

Southern blot and Northern blot analyses

Genomic DNA was isolated from individual mouse tails, and then was blotted onto nylon membranes according to standard protocols. Total RNA was extracted from liver tissues using ISOGEN (Nippon Gene, Tokyo, Japan), denatured with formamide and blotted onto nylon membranes. The mouse cDNAs described above were labeled with (α-^{32}P) dCTP using a Megaprime DNA labeling kit (Amersham Life Science, Tokyo, Japan) and then were used for hybridization[16,17].

RESULTS

An expression unit was constructed that contained the entire HDGF cDNA under the control of the mouse albumin promoter/enhancer (Alb-HDGF, Figure 1). Purified fragments were used for pronuclear injection and potential founders were analyzed for the genomic integration(s) of the transgene. Three founders containing the Alb-HDGF sequence were identified by Southern blot, and the transgenes were successfully transmitted in two lines (Figure 2A: Tg-48, and Tg-21). Northern blot analysis revealed that HDGF was highly expressed in the adult liver of Tg-48 mice, whereas HDGF expression in the liver of Tg-21 mice was almost equal to the wild-type mice (Figure 2B). We therefore used the Tg-48 mice to analyze the effects of HDGF overexpression in hepatocytes.

HDGF overexpressing mice (Tg-48) developed normally and did not show any abnormality in appearance. In addition, no obvious histological abnormality associated with the expression of HDGF was detected in these mice up to 12 mo of age (data not shown). The expression patterns of genes related to hepatocyte differentiation were investigated by Northern blotting to examine the effects of HDGF overexpression on liver development in detail. In normal mice, consistent with the previous studies, G-6-

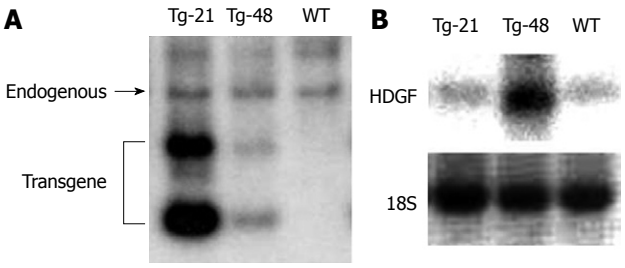

Figure 2 Genomic integration and mRNA expression of the HDGF-transgene. A: Southern blot analysis with HDGF cDNA probe. Genomic DNA was isolated from individual mouse tails and Southern blot analysis was performed according to standard methods. The bands representing endogenous HDGF and transgenes are shown. Genomic DNA of a transgenic mice (line number 21: Tg-21) contains high copy numbers of the transgene. Genomic DNA of the other transgenic mice (line number 48: Tg-48) contains low copy numbers of the transgene; B: Northern blot analysis with HDGF cDNA probe. Total RNA was isolated from liver tissues of both transgenic (Tg) and wild-type (WT) mice. Twenty micrograms of total RNA extracted was loaded and hybridized with mouse cDNA of the HDGF-specific sequence. HDGF expression was high in the liver of the Tg-48 mouse. The expression level of HDGF in the liver of Tg-21 was almost equal to the level expressed in the control liver. Ribosomal RNA of 18S is shown in the lower panel.

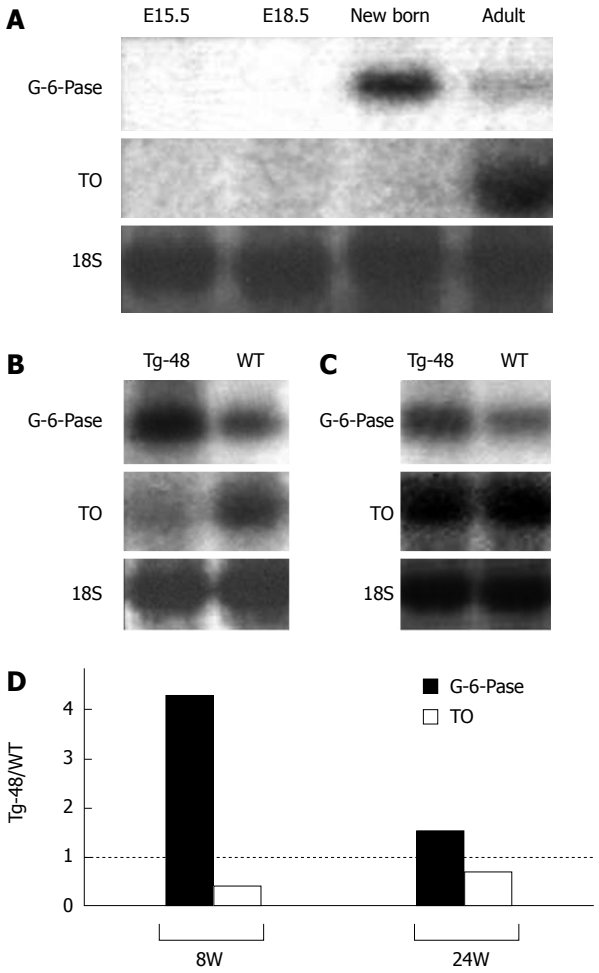

Figure 3 The expression of differentiation marker genes of hepatocytes. A: The expression of differentiation marker genes of hepatocytes in normal mice. RNA was extracted from fetal mice of E (embryonic day) 13.5 and 15.5, and postnatal mice of zero weeks (new born) and 8 wk after birth. Twenty micrograms of total RNA was loaded and hybridized with mouse cDNA probes. Ribosomal RNA of 18S is shown in the lowest panel; B: The expression of differentiation marker genes of hepatocytes in adult (8 wk) transgenic mice. In the control liver of 8-wk-old mouse, G-6-Pase expression is low and TO expression is high. In contrast, G-6-Pase expression is high and TO expression is low in the transgenic liver. Ribosomal RNA of 18S is shown in the lowest panel; C: The expression of differentiation marker genes of hepatocytes in adult (24w) transgenic mice. Unlike the 8-wk-old mouse, expression levels of G-6-Pase and TO show small differences between the transgenic liver and the normal liver. Ribosomal RNA of 18S is shown in the lowest panel. D: Densitometry measurements of Northern blot analysis. Northern blot signals in Figure 3B and 3C were measured and normalized by the internal control bands (18S). The expression levels of G-6-Pase and TO in the liver of Tg-48 mouse were compared with the levels in the control liver. The ratios of band intensities (Tg-48/WT) were graphically shown. Two independent experiments were conducted in duplicate, and similar results were obtained.

Pase expression was high in the neonatal liver and relatively low in adult (8 wk old) liver, whereas TO expression was higher in the adult liver in comparison to the neonatal liver (Figure 3A). Conversely, the expression of G-6-Pase was high, while the TO expression was low in the adult livers of transgenic mice (Figure 3B and D). These gene expression patterns observed in the livers of adult transgenic mice, were similar to the patterns observed in the neonatal stage of control livers. These findings have indicated that HDGF overexpressing hepatocytes in adult (8 wk old) transgenic mice appear to have the characteristics of hepatocytes in neonatal wild-type mice, suggesting the possible maturational retardation of hepatocytes in the transgenic mice. However, G-6-Pase expression in the transgenic liver in 24 wk-old mice decreased to the level of the normal liver, and the expression level of TO in the transgenic liver was increased almost to the level of the control liver, although small differences were still observed (Figure 3C and D). Therefore, HDGF overexpression did not completely block hepatocyte maturation and HDGF overexpressing hepatocytes could acquire almost fully differentiated phenotypes. These findings suggest that HDGF-overexpression partially suppresses the hepatocyte differentiation observed in the post-natal stage and thus causes the maturational delay of the hepatocytes.

DISCUSSION

A number of studies have suggested that HDGF is involved in the development of various organs[10,11,16,18,21]. We have demonstrated that HDGF is a unique growth factor, which is highly expressed in fetal liver and promotes fetal hepatocyte proliferation[16]. However, familial genes often compensate for the functions of other family members, and HDGF-null mice have been reported to show no obvious phenotype, perhaps as a result of the redundant functions of HDGF related genes[22]. We therefore generated the HDGF transgenic mice and examined the functional role of HDGF *in vivo* according to the gain-of-function method.

Although several other groups have also reported the involvement of HDGF in the development of various organs through its growth stimulating activity, little is known about the role of HDGF in cellular maturation. As for hepatocyte differentiation, Kamiya *et al*[20] established a primary culture system of murine fetal hepatocytes to investigate the mechanism that controls late fetal liver

development. In the culture system, the administration of Oncostain M and dexamethasone can induce hepatocyte differentiation and recapitulate the maturational process of hepatocytes ranging from mid-gestation to new-born stage. This culture system was used to clarify the involvement of HDGF in hepatocyte differentiation although down-regulation of HDGF could not induce the cellular differentiation process of the late gestation stage[16].

In the present study, the overexpression of HDGF under the control of the albumin promoter did not cause any apparent morphological abnormalities in the liver. However, the gene expression patterns showed the possibility that the maturational process of hepatocytes during the post-natal stage was disturbed. This result is consistent with the report by Lepourcelet *et al*[18], which documented that overexpression of HDGF in the mouse fetal gut explants retards epithelial differentiation, suggesting a suppressive role of HDGF in epithelial differentiation.

Several proteins strongly expressed in both tumors and fetal organs, such as carcinoembryonic antigen and AFP, are known as oncofetal proteins[23,24]. HDGF is expressed exclusively in both fetal and cancer tissues, indicating that HDGF can also be regarded as an oncofetal protein. Although several oncofetal proteins are clinically used as tumor markers, there are few proteins whose functional roles in cancer cells have so far been demonstrated. HDGF expression is strongly associated with the prognosis of many malignant diseases including pancreatic cancer, esophageal cancer, colorectal cancer, gastrointestinal stromal tumor, gastric cancer and heptocellular carcinoma (HCC)[25-31]. Recently, Lee *et al*[32] showed that individuals with HCC who shared a gene expression pattern with fetal hepatoblasts had a poor prognosis. The gene expression pattern that distinguished this subtype from other types of HCC contained the markers of oval cells (hepato-cholangio progenitor cells), thus suggesting that the HCC of this subtype may be derived from hepatic progenitor/stem cells. Two groups have shown that high expression of HDGF is closely related to the poor prognosis of HCC[30,31] and HDGF stimulates the growth of immature fetal hepatocytes[16]. Recently, we have found that HDGF is highly expressed in oval cells and promotes their proliferation (Iwamoto et al. in preparation), thereby suggesting the involvement of HDGF in the proliferation of immature hepatic cells. Therefore, HDGF may stimulate the proliferation of HCC cells derived from hepatic progenitor/stem cells and thereby cause the poor prognosis. HDGF expression may maintain the characteristics of immature cells and be associated with high growth activity of malignant cells. HDGF not only promotes hepatocyte proliferation but also inhibits their differentiation, indicating that HDGF is an oncofetal protein which participates both in the cellular growth and differentiation. Clarifying the functional role of HDGF would give us new insights into molecular mechanisms common to normal and malignant hepatic cell growth.

Since HDGF-null mice did not show any remarkable abnormalities, perhaps as a result of the compensation by HDGF-related genes, the down-regulation of HDGF should inhibit the growth of cancer cells without any serious side effects on normal organs. Therefore, HDGF is considered to be a candidate therapeutic target. Although little is known about the regulation of HDGF expression, we recently have found that Vitamin K2 negatively controls the transcription of HDGF in hepatoma cells[33]. However, the suppressive effects of the Vitamin K2 are limited and it is necessary to elucidate the whole regulation of the HDGF expression in hepatic cells, especially in hepatoma cells.

In conclusion, HDGF overexpressing transgenic mice showed the possible inhibitory role of HDGF on hepatocyte differentiation. The identification of both the regulation and signal transduction of HDGF makes it possible to obtain a better understanding of liver development, regeneration, and carcinogenesis.

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COMMENTS

Background

During liver development, immature hepatocytes differentiate and acquire many metabolic functions. However, few studies have documented the growth and differentiation of post-natal hepatocytes.

Research frontiers

Hepatoma-derived growth factor (HDGF) is a heparin-binding protein, which is involved in the hepatocyte proliferation during liver development. However, the role of HDGF in hepatocyte differentiation has not been elucidated. In this study, the authors show the inhibitory role of HDGF in the hepatocyte maturation by use of the transgenic mice.

Innovations and breakthroughs

In this article, transgenic mice were established which overexpressed HDGF in hepatocytes under the transcriptional control of the mouse albumin promoter/enhancer. This is the first report about the transgenic mouse of HDGF. Furthermore, this is the first study to clarify the functional role of HDGF in the hepatocyte maturation.

Applications

The results show the possibility that HDGF may maintain the characteristics of immature cells with high growth activity. This study might represent a future strategy for the prevention or treatment of the diseases by the targeting of HDGF.

Terminology

HDGF is a heparin-binding acidic glycoprotein consisted of 240 amino acids, which is identified from the conditioned media of HuH-7 hepatoma cells. HDGF plays an important role in the organ development and tissue repair. HDGF has been demonstrated to be a unique growth factor involved in liver development, regeneration and carcinogenesis.

Peer review

On the whole, this study holds novelty and importance in understanding the process of hepatocyte maturation. While the study seemed well conducted, it is not sure from the text whether repeated analysis of Western blot has been conducted and to what extent the G-6-Pase and TO expressions were raised or dropped with time. I suggest densitometry measurements of repeated Western blot analysis and to provide a bar chart on the normalized readings.

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CASE REPORT

Synchronous development of HCC and CCC in the same subsegment of the liver in a patient with type C liver cirrhosis

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Abstract

As a result of having undergone computed tomography (CT), a 75-year-old woman with type-C liver cirrhosiswas shown to have two tumors on the ventral and dorsal sides of subsegment 3 (S3). The tumor on the ventral side was diagnosed as a classic hepatocellular carcinoma (HCC), while that on the dorsal side was considered atypical for a HCC. Although the indocyanine green (ICG) findings indicated poor hepatic reserve, the prothrombin time (PT) was relatively good. An operation was performed in February 2007; however, this resulted in exploratory laparotomy. Dynamic CT performed 12 mo after the operation revealed that the tumor on the dorsal side of S3 had apparently increased. The marginal portion of the tumor was shown to be in the early and parenchymal phases, while the internal portion was found to have grown only slightly in the delayed phase. We diagnosed this tumor as a cholangiocellular carcinoma (CCC). S3 subsegmentectomy was performed in April 2008. The tumor on the ventral side was pathologically diagnosed as a moderately differentiated HCC, and that on the dorsal side was diagnosed as a CCC. We can therefore report a rare case of synchronous development of HCC and CCC in the same subsegment of the liver in a patient with type-C liver cirrhosis. We also add a literature review for all the reported cases published in Japan and around the world, and summarize the features of double cancer exhibiting both HCC and CCC.

Key words: Double cancer; Hepatocellular carcinoma; Cholangiocellular carcinoma; Synchronous; Literature review

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Watanabe T, Sakata J, Ishikawa T, Shirai Y, Suda T, Hirono H, Hasegawa K, Soga K, Shibasaki K, Saito Y, Umezu H. Synchronous development of HCC and CCC in the same subsegment of the liver in a patient with type C liver cirrhosis. *World J Hepatol* 2009; 1(1): 103-109 Available from: URL: http://www.wjgnet.com/1948-5182/full/v1/i1/103.htm DOI: http://dx.doi.org/10.4254/wjh.v1.i1.103

INTRODUCTION

The incidence of synchronous development of hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CCC) (combined HCC and CCC) has been reported to be low (constituting between 0.54% and 0.70% of primary liver cancers)[1-3]. Allen and Lisa defined three types of HCC-CCC: (a) separate masses composed of either hepatocellular or cholangiocellular components (mixed type); (b) contiguous but independent masses of hepatocellular and cholangiocellular components (combined type); and (c) an intimate intermingling of hepatocellular and glandular elements (double cancer)[4]. A literature search of Japana Centra Revuo Medicina found 18 cases[5-22] of synchronous double cancer with HCC and CCC, while a search of MEDLINE found only 16 such cases[13,23-36] (including 13 cases reported in Japan). In this report, we describe a rare case of synchronous development of HCC and CCC in the same subsegment of the liver in a patient with type-C liver cirrhosis. We also review all the previously reported cases of synchronous double cancer.

CASE REPORT

A 75-year-old woman diagnosed with chronic hepatitis or liver cirrhosis (type C) had been followed as an outpatient since 1982. Although endoscopic variceal ligation and endoscopic injection sclerotherapy (EIS) had been carried out several times, the esophageal varices ruptured in December 2002. Five EIS treatments were thenfollowed, after which the condition of the patient maintained static, that is, without the esophageal varices showing red.

Dynamic computed tomography (CT) of the liver performed on December 18, 2006, revealed two tumors, each 1 cm or less in diameter, on the ventral and dorsal sides of S3 (Figure 1A-C). The patient was hospitalized for further examination and therapy in January 2007. We could not detect the tumors by ultrasonography and were unable to perform a tumor biopsy. With abdominal angiography, the left hepatic arterial angiogram (LHAG) showed no tumor stain on S3 and the superior mesenteric arterial angiogram (SMAG) showed a large shunt through the epigastric vein during the portal phase (Figure 1D). CT during arterial portography showed a 1 cm defect on the ventral side of S3; however, no defect was evident on the dorsal side (Figure 1E). Because CT had shown the tumor on the ventral side of S3 to be densely enhanced in the early phase and washed out in the delayed phase, we diagnosed the tumor as a classic HCC. The values for indocyanine green (ICG) dye retention at 15 min (R15) and elimination (K) were 32.0% and 0.070, respectively. However, because prothrombin time (PT) was 82.7%, indicating good hepatic reserve, the operation was performed in February 2007. Since the epigastric veins were overdeveloped, the liver appeared very cirrhotic and we were unable to detect the two masses by intraoperative ultrasonography. The first operation therefore resulted in exploratory laparotomy.

Dynamic CT of the liver performed 12 mo after the first operation revealed the tumor on the ventral side of S3 to be a classic HCC. Furthermore, the tumor on the dorsal side of S3 had apparently increased to 3 cm or less in diameter. It was discovered that the peripheral portion of the tumor showed typical signs of early and parenchymal phases, while the internal portion was slightly and gradually enhanced in the delayed phase. The peripheral portion of the tumor was typically found to be enhanced in the early and parenchymal phases, while the internal portion was found to be slightly and gradually enhanced in the delayed phase. (Figure 2A-C). Subcutaneous ultrasonography of the abdomen revealed a hyperechoic mass, 14 mm in diameter, at the site of the tumor on the ventral side, a finding characteristic of HCC rich in fat (Figure 2D). The tumor on the dorsal side of S3 was also represented by a hyperechoic mass, 30 mm or less in diameter, with irregular and unclear margins, but this finding was not typical for HCC (Figure 2D). In abdominal angiography, the LHAG showed no tumor stain and because the shunt through the epigastric vein was resected at the first operation, the SMAG did not show this shunt. 12 mo after the first operation, the patient's platelet count was 8.4 $\times 10^4/mm^3$. The ICG R15 and K values were 35.9% and 0.066, respectively; however, PT was 81.3%. Although the ICG findings indicated poor hepatic reserve, PT was relatively good. We diagnosed the tumor on the ventral side of S3 as HCC and that on the dorsal side of S3 as a CCC tumor. Because of the poor hepatic reserve, lateral segmentectomy was ruled out.

S3 subsegmentectomy was performed in April 2008 (Figure 3A and B). The tumor on the ventral side was pathologically diagnosed as a moderately differentiated HCC (with nodular, trabecular, and plate-like components) (Figure 3C) and that on the dorsal side was diagnosed as CCC (diffuse type showing a vestigial remnant of the tumor) (Figure 3D). Pathological examination also confirmed that the larger CCC tumor on the dorsal side was partially in contact with the HCC tumor on the ventral side of S3 (Figure not shown). We administered two courses of chemotherapy with gemcitabine hydrochloride (Gemzar®, Eli Lilly Japan, Kobe, Japan) followed by oral administration of S-1 (TS-1®, Taiho Pharma, Tokyo, Japan).

DISCUSSION

The present report describes a case of synchronous development of HCC and CCC in the same subsegment of the liver. Only three similar cases[19,22,30] have been previously reported, and this condition is considered to be very rare. According to several earlier reports[37-39], hepatitis C virus (HCV)-related chronic hepatitis and cirrhosis are major risk factors for both HCC and CCC. The patient described in this report was found to be positive for HCV.

Earlier research has suggested the existence of amphi-potential progenitor cells that can differentiate into hepatocytes as well as cholangiocytes[40].

The following points may explain the mechanism un-

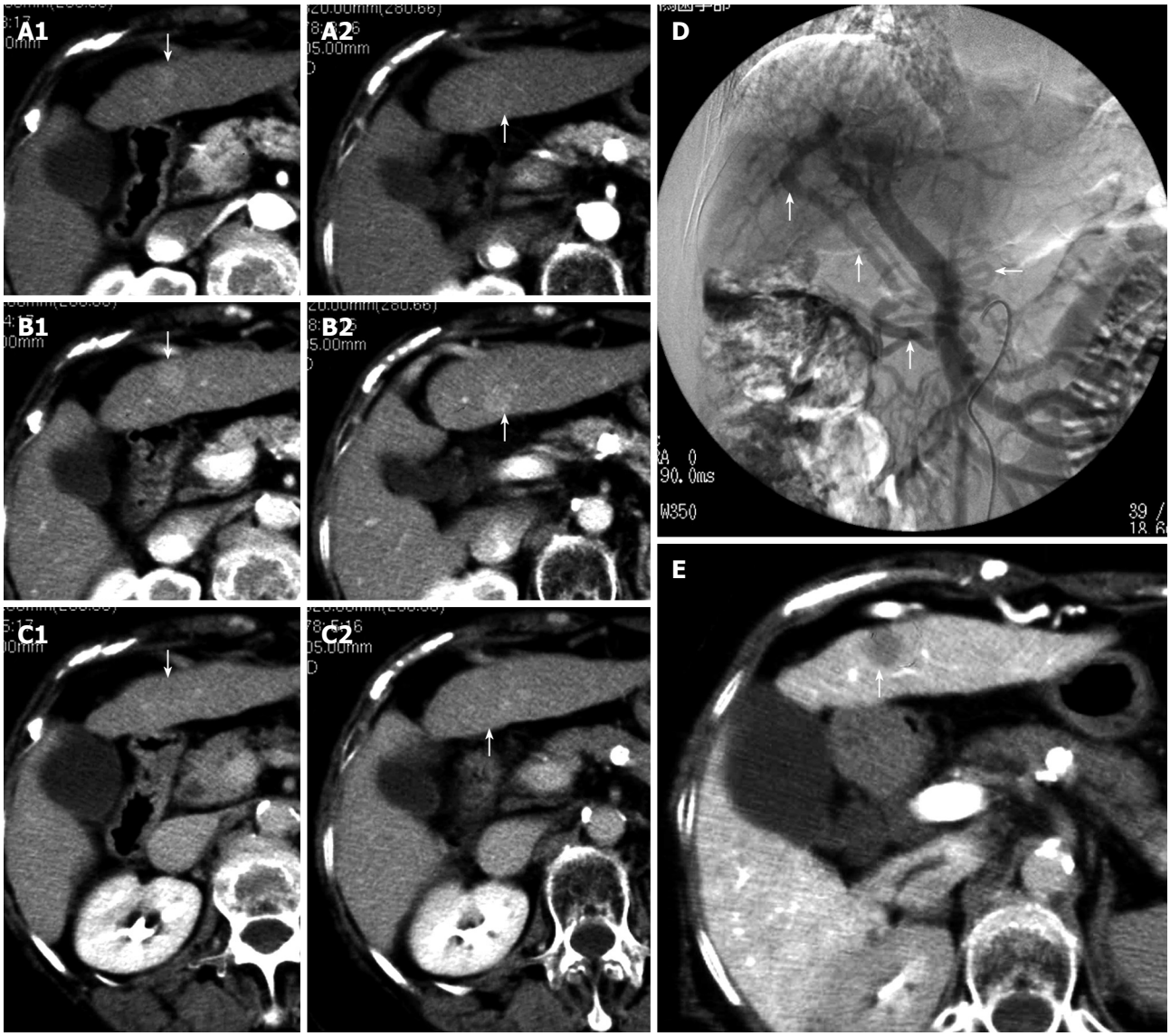

Figure 1 Dynamic computed tomography findings at the onset of double cancer with hepatocellular and cholangiocellular carcinomas. Two tumors, ~1 cm in diameter, were detected on the ventral and dorsal sides of S3. Arrows indicate the tumors (A1, A2 arterial phase; B1, B2 parenchymal phase; C1, C2 delayed phase). Angiographic findings at the onset of double cancer; D: A superior mesenteric arterial angiogram showing a large shunt through the epigastric vein during the portal phase (the arrows); E: Computed tomography during arterial portography (CTAP) showing a ~1 cm defect on the ventral side of S3 (the arrow), which was diagnosed as a classic hepatocellular carcinoma. No defect can be seen on the dorsal side of S3.

derlying the development of combined HCC and CCC: (1) during the differentiation of the tumor into the two cancer phenotypes HCC and CCC, a trigger may cause the amphi-potential progenitor cells to turn malignant, undergo proliferation, and become tumorous in the same lesion, and (2) the cells that have already differentiated and matured into hepatocytes and cholangiocytes may become malignant and undergo proliferation in the presence of chronic liver inflammation. In the present case, although the cells involved belonged to the same subsegment of the liver, these cells could have metachronously turned malignant, undergone proliferation, and become tumorous, resulting in a synchronous double cancer (HCC-CCC) by either of the mechanisms described above.

In the present case, on performing dynamic CT in December, 2006, HCC and CCC were observed as being situated far from each other. However, we had pathologically confirmed that the larger CCC on the dorsal side was partially in contact with the HCC on the ventral side of S3 in the resected specimen, thus demonstrating that these tumors constituted a combined type of HCC-CCC, which had developed from different lesions in the liver. In this paper we have described the entire course of the two tumors involved in a combined type of HCC-CCC, making these findings valuable for research as well as clinical purposes.

The usual gross appearance of a mass-forming CCC is a large, white, firm tumor that is solid and fibrous, with a sclerotic appearance on the cut surface of the specimen, accompanied by a frequent finding of dense fibrous stranding in the central portion[41]. In most cases of mass-forming CCCs, ultrasonography reveals a hypoechoic mass with satellite nodules around the tumor[42]. However, the echo patterns are diverse and non-specific[43]. Dynamic

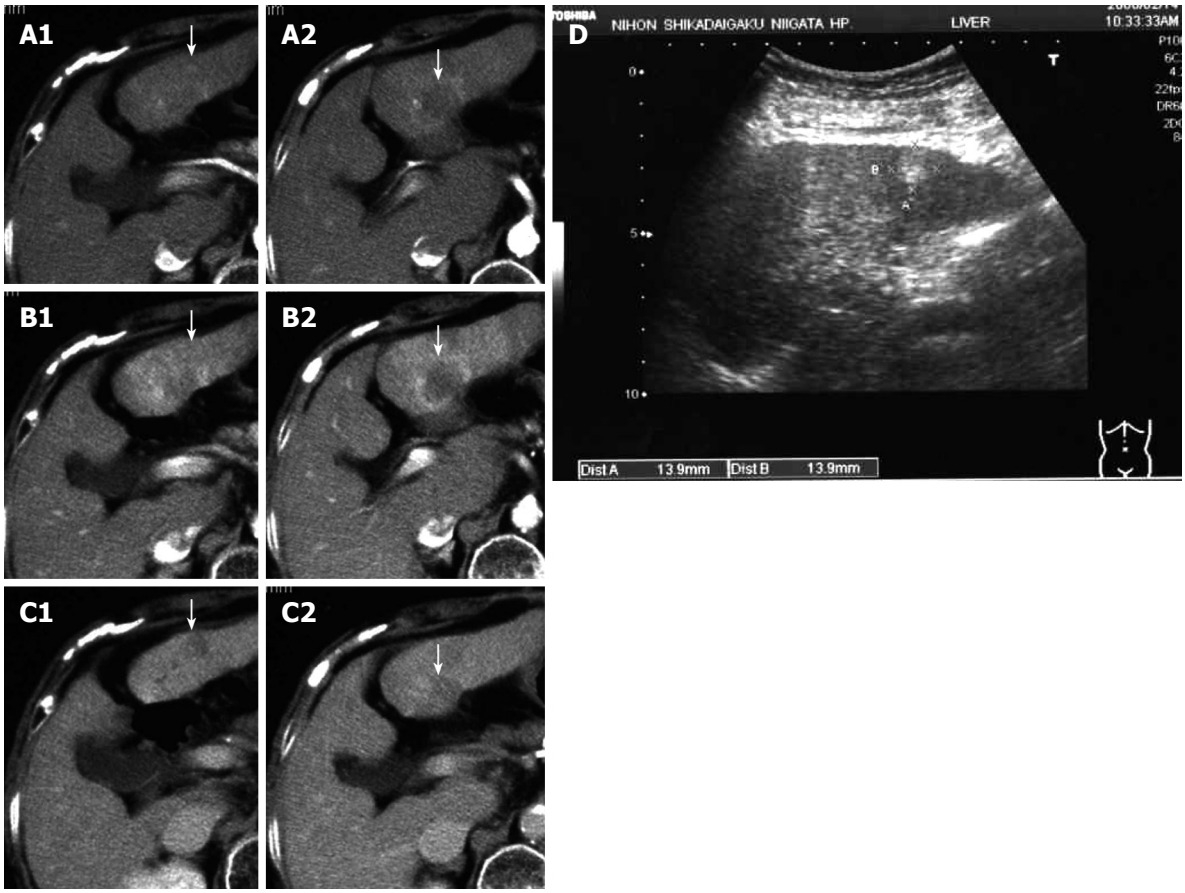

Figure 2 Dynamic computed tomography performed 12 mo after the first operation. The tumor on the ventral side of S3 appears to be a classic hepatocellular carcinoma and that on the dorsal side of S3 appears to be increased to ~3 cm in diameter. Typical findings including enhancement of the peripheral portion of the tumor in the early (A1) and parenchymal (B2) phases, and the slight and gradual enhancement of the internal portion in the delayed phase were observed (C2). Arrows indicate the tumors (A1, A2 arterial phase; B1, B2 parenchymal phase; C1, C2 delayed phase). Subcutaneous ultrasonography performed 12 mo after the first operation (D); The tumor on the ventral side of S3 is represented by a hyperechoic mass, 14 mm in diameter, a finding characteristic of hepatocellular carcinoma rich in fat. The tumor on the dorsal side of S3 is also represented by a hyperechoic lesion, ~30 mm in diameter, with irregular and unclear margins. Arrows indicate the tumors (D).

CT and magnetic resonance imaging (MRI) reveal rim-like or band-like peripheral contrast enhancement of variable thickness around the tumor during the early phase, with progressive and concentric filling of the contrast material at a later phase[44]. CCCs appear hypointense on T1-weighted images and hyperintense on T2-weighted images[45,46].

In the present case, the size of the CCC was small (1 cm in diameter) when first discovered. We could not detect the tumor by ultrasonography because of overdeveloped epigastric veins, and were unable to identify the typical findings of an enhanced marginal portion in the early phase and the gradual enhancement of the internal portion in the delayed phase. The existence of this tumor was therefore questionable. However, abdominal ultrasonography performed 12 mo after the first operation showed a heterogeneously hyperechoic area with irregular margins on the dorsal side of S3. Dynamic CT of the liver performed during the same period showed a rim-like or band-like enhancement around the tumor in the early phase and a slight enhancement of the central portion of the tumor in the delayed phase. We preoperatively diagnosed the tumor on the dorsal side of S3 as a CCC. Early diagnosis of CCCs is very important for an improvement in prognosis. We hope that imaging studies will eventually be conducted for preoperative diagnosis of CCCs that are $\leqslant$ 1 cm in diameter.

In the present case, however, because of progressive liver cirrhosis, with poor hepatic reserve and an enlarged lateral segment of the liver, we performed an S3 subsegmentectomy. Because type-C liver cirrhosis generally results in poor hepatic reserve, it is imperative to consistently monitor the development of not only HCCs, but also CCCs, and facilitate early detection and treatment by curative hepatectomy.

Literature review of reported synchronous double cancer cases with HCC and CCC

Synchronous double cancer is particularly rare in the case of combined HCC and CCC. A literature search of Japana Centra Revuo Medicina database, version 4 (systematic literature search system through a computer web site for Japanese literature) found 18 cases[5-22] of synchronous double cancer with HCC and CCC, while a search of MEDLINE found only 16 such cases[13,23-36] (including 13 cases reported in Japan). The Japana

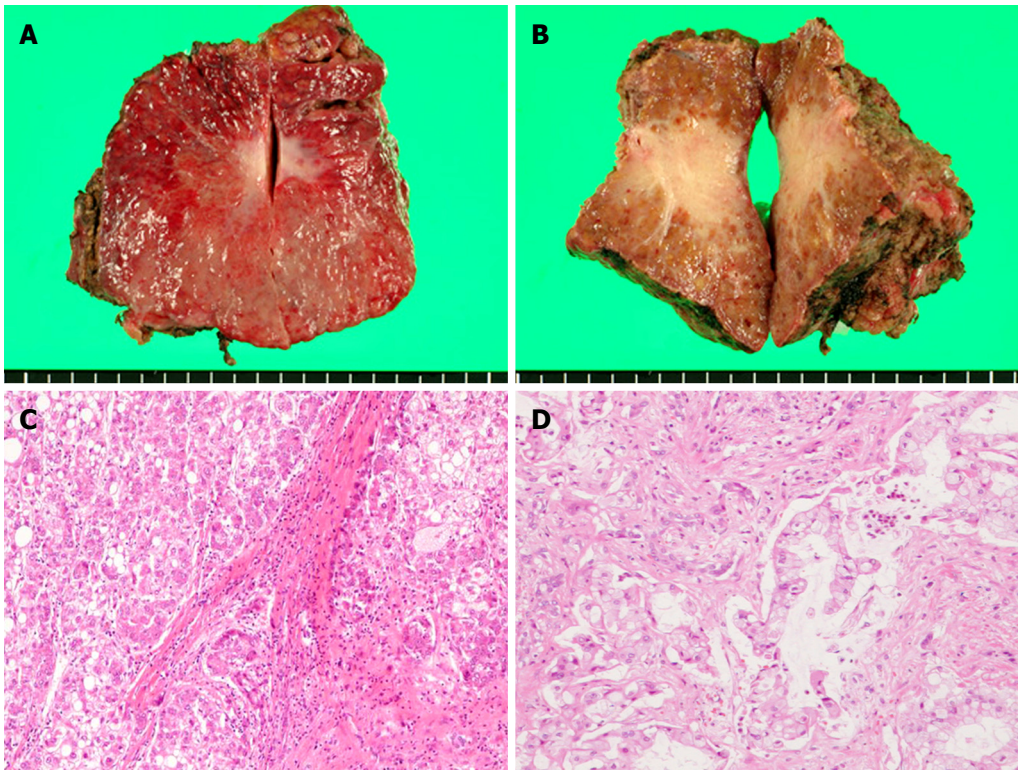

Figure 3 Gross findings of the resected S3 subsegment. A: The cut surface of the tumor on the ventral side of S3; B: The cut surface of the tumor on the dorsal side of S3. Histopathological findings of the two resected tumors; C: The tumor on the ventral side was pathologically diagnosed as a moderately differentiated hepatocellular carcinoma (HCC) (with nodular, trabecular, and plate-like components); D: The tumor on the dorsal side was pathologically diagnosed as a cholangiocellular carcinoma (CCC) (diffuse type showing a vestigial remnant of the tumor).

Centra Revuo Medicina database available for analysis has a good reputation for accuracy and completeness. We review all 33 cases in the following section (Table 1). (A literature search of both Japana Centra Revuo Medicina and MEDLINE found one duplicated case, which Yoshikawa *et al*[13] have reported).

Since Mitsui's report in 1986[5], a total of 33 cases of synchronous double cancer with HCC and CCC have been reported in MEDLINE and Japana Centra Revuo Medicina databases. The patients were aged 66.7 ± 7.4 years (mean ± SD) and the male to female ratio was 30:3. Twenty-four out of 33 cases (72.7%) were positive for HCV and many double cancers developed in livers with HCV infections. Three out of 33 cases (9.4%) were positive for HBs antigen and about 10% of double cancers developed in livers with HBV infections. Two out of 33 cases (6.1%) had neither HBV nor HCV infection, while one had both HBV and HCV infections. Double cancer with HCC and CCC tended to develop in livers with HCV infection, followed by HBV infection, as in HCC. In contrast, Zhang *et al*[47] reported that 7 out of 12 cases (58.3%) of the combined type (or mixed type) of HCC and CCC were positive for HBs antigen, but no cases were positive for HCV. It is suggested that the background of double cancer is distinctly different from that of the combined (or mixed) type HCC and CCC. High levels of serum carcinoembryonic antigen (CEA) (> 3.0 ng/mL), CA19-9 (> 37 ng/mL), and α fetoprotein (AFP) (> 10 ng/mL) were detected in 11 of 24 cases (45.8%), 12 of 21 cases (57.1%), and 25 of 33 cases (75.8%) (except unknown cases), respectively. Regarding the localization of HCCs and CCCs, only 3 cases of double cancer that developed in the same subsegment of the liver have been reported. In addition, 5 out of 33 cases (15.2%) of double cancer revealed synchronous development in the segments of the liver accessed by the same portal vein (for example, in S5 and S8, or in S2 and S3). In the cases of these double cancers, it is conceivable that (1) the amphi-potential progenitor tumor cells disseminated to a different subsegment in the same portal vein-accessed segment of the liver *via* the portal vein before they differentiated to either HCC or CCC, and double cancer (HCC-CCC) developed in the same segment of the liver or that (2) in a different subsegment in the same portal vein-accessed segment of the liver, hepatocytes and cholangiocytes with HCV infection became cancerous and underwent proliferation. Twenty-two of 33 cases (66.7%) with double cancer exhibited synchronous development in a different portal vein-accessed segment of the liver. In these cases, it is conceivable that the second mechanism was responsible for the development of the double cancer (multicentric development of HCC). Further laboratory studies are needed to clarify and explain the mechanisms of development of synchronous double cancer.

Across the 33 previously reported cases, the mean maximum size of the HCC and CCC tumors (if the tumors were multiple) were 3.9 ± 2.7 cm and 3.3 ± 3.0 cm,

Table 1 Clinical status of reported cases of synchronous double cancer with hepatocellular carcinoma and cholangiocellular carcinoma

Clinical status	Compiled numbers
Age, in years (mean ± SD) (range)	66.7 ± 7.4 (51-84) (y/o)
Sex	Total: 33
Male	30 (90.9%)
Female	3 (9.1%)
Anti-HCV	Total: 33
Positive	24 (72.7%)
Negative	9 (27.3%)
HBs antigen	Total: 33
Positive	3 (9.1%)
Negative	30 (90.9%)
	Total: 33
Neither HBV nor HCV infection	2 (6.1%)
	Total: 33
Both positive HBV and HCV infections	1 (3.0%)
Tumor markers	The number/Total number
High levels of CEA (> 3.0 ng/mL)	11/24 (45.8%)
High levels of CA19-9 (> 37 ng/mL)	12/21 (57.1%)
High levels of AFP (> 10 ng/mL)	25/33 (75.8%)
Localization of HCCs and CCCs	Total: 33
In the same subsegment	3 (9.1%)
In the segment of the liver accessed by the same portal vein	5 (15.2%)
In a segment of the liver accessed by a different portal vein	22 (66.7%)
Mean maximum size of the tumors (mean ± SD) (range)	Total: 33
HCC	3.9 ± 2.7 (0.8-10.0) (cm)
CCC	3.3 ± 3.0 (0.6-14.0) (cm)
Pathological diagnosis of the non-cancerous portions of the liver	Total: 33
Chronic hepatitis	19 (57.6%)
Liver cirrhosis	12 (36.4%)
Therapeutic procedures	Total: 33
Surgery	24 (72.8%)
TAE	2 (6.1%)
Hepatic arterial infusion therapy	1 (3.0%)
Surgery plus PEIT	1 (3.0%)
Surgery plus MCT	1 (3.0%)
Liver transplantation	1 (3.0%)
Unknown	3 (9.1%)

y/o: Years old; HCV: Hepatitis C virus; HB(V): Hepatitis B (virus); CEA: Carcinoembryonic antigen; AFP: α fetoprotein; HCC: Hepatocellular carcinoma; CCC: Cholangiocellular carcinoma; TAE: Transcatheter arterial embolization; PEIT: Percutaneous ethanol injection therapy; MCT: Microwave coagulation therapy.

respectively. According to the pathological diagnosis of the non-cancerous portions of the liver, 19 cases (57.6%) were diagnosed with chronic hepatitis and 12 (36.4%) with liver cirrhosis. Double cancers were more likely to develop from chronic hepatitis than from liver cirrhosis. We think that the cases that undergo appropriate examinations and successful treatments because of their higher hepatic reserve tended to get reported and documented. There is thus a possibility of bias in the selection of the reported cases. Most such cases underwent surgery (24 out of 30 cases or 80.0%); the other treatment techniques performed included transcatheter arterial embolization, hepatic arterial infusion therapy, surgery plus percutaneous ethanol injection therapy, and surgery plus microwave coagulation therapy and liver transplantation. The surgical procedure is planned according to the hepatic reserve; many cases of double cancer with HCC and CCC were treated surgically by curative resection.

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Events Calendar 2009

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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/eid.htm

Patent (list all authors)

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

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