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12 がん免疫療法

はじめに(近年のがんワクチン療法の進展)

近年，がん治療の選択肢の一つとして，比較的副作用の少ない免疫療法が注目されている．本年(2009年)の米国臨床腫瘍学会(ASCO)では，無治療進行濾胞性リンパ腫における化学療法後完全寛解例に対して，自己リンパ腫由来イディオタイプ蛋白ワクチン治療の無作為化二重盲検比較第3相試験の結果，ワクチン群が6カ月以上の寛解状態を維持でき，コントロール群に比べ，有意に無再発生存期間の延長がみられたと報告された[1]．また，進行悪性黒色腫に対する大量IL-2併用改変gp100ペプチドワクチン治療の前向き無作為第3相臨床試験の結果，ペプチドワクチン投与群で有意に高い奏効率と，無増悪生存期間の延長がみられ，ペプチドワクチン療法としては，第3相臨床試験で初めて有意な改善を示す報告がなされた[2]．海外では，各種がんに効果的な腫瘍抗原ペプチド，蛋白，遺伝子，ウイルスベクター，患者自己腫瘍由来抗原蛋白，がん抗原感作樹状細胞を利用したがんワクチンの開発が精力的に行われ，臨床試験が進められている．本稿では，海外にて後期臨床試験まで進められている主ながんワクチン臨床試験と国内のがんワクチン臨床試験について，本年のASCOで報告された最新の情報も含めてがん種別に述べてみたい．

1．免疫療法について

がん免疫療法は，大きく4種類に分類できる(表1)．抗原非特異的能動免疫療法の一つであるIFN-α療法やIL-2療法などのサイトカイン療法は，すでに腎細胞がんで実施されている．抗原非特異的受動免疫療法の一つであるLAK療法は，IL-2で活性化したリンパ球を体外で大量培養し，体内に戻す細胞移入療法である．

1991年にベルギーのBoonらが，世界で初めてがん抗原MAGE-1を同定して以来[3]，多くのがん抗原が次々に同定され，がんに特異的な抗原を標的とした免疫療法の開発が始まった．抗原特異的能動免疫療法には，がん抗原由来のペプチド，蛋白，DNAなどをアジュバントとともに投与し，体内で抗原提示細胞を感作し，エフェクター細胞であるキラーT細胞やヘルパーT細胞を活性化，増殖させるワクチン療法と，体外で患者由来の抗原提示細胞である

表1　がん免疫療法の分類

	能動免疫	受動免疫
抗原特異的	ワクチン療法(ペプチド，蛋白，遺伝子，ウイルスベクター) 抗原感作樹状細胞療法	抗体療法 抗原特異的養子免疫細胞療法
抗原非特異的	サイトカイン療法	LAK療法

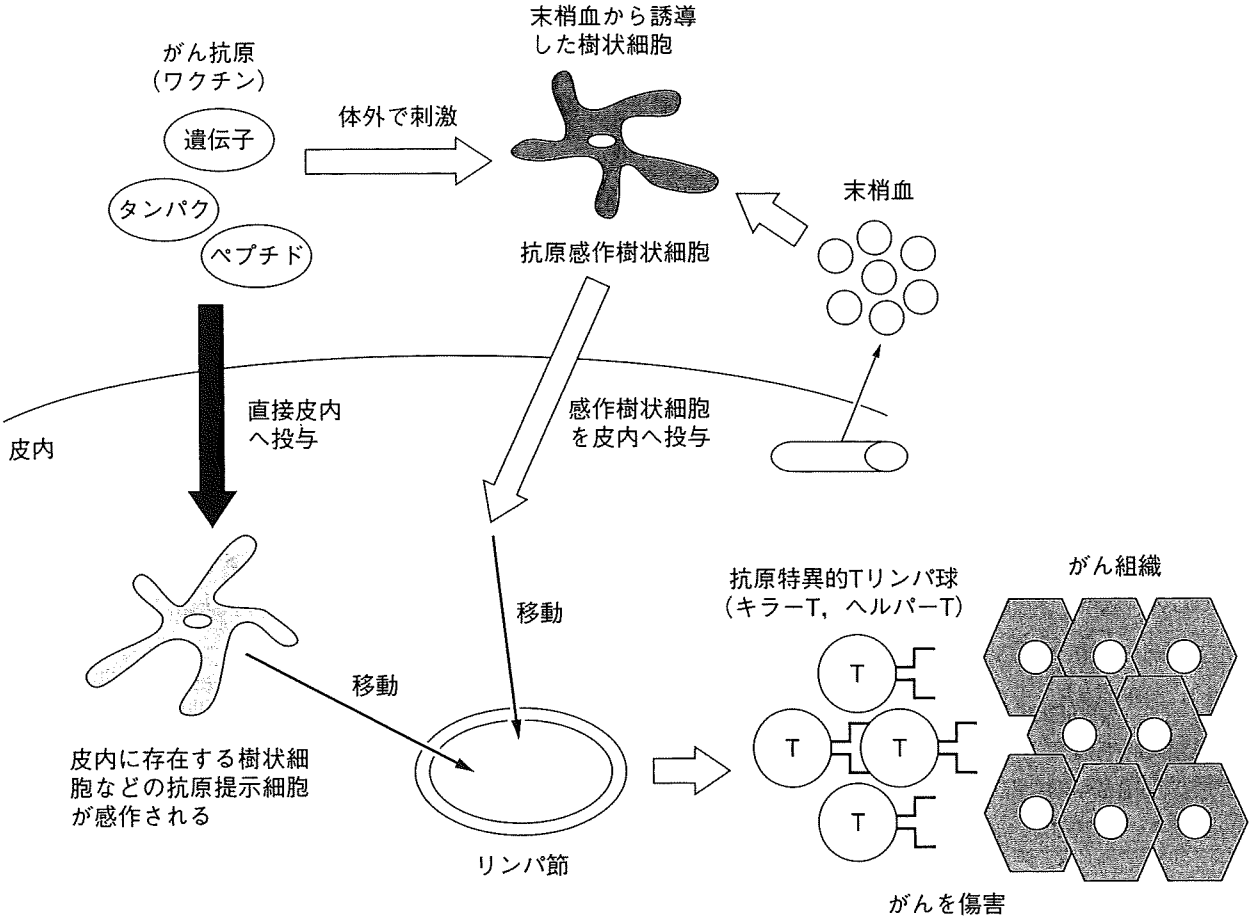

図 1　がんワクチン療法　概念図

樹状細胞を培養し，がん抗原を感作した後，体内へ戻す抗原感作樹状細胞療法がある(図 1)．近年精力的に臨床試験が行われ，最も研究が進んでいる悪性黒色腫では第 3 相臨床試験での有意に高い奏効率と，無増悪生存期間の延長も証明された[2]．さらに抗原特異的受動免疫療法として，米国国立衛生研究所の Rogenberg らは，悪性黒色腫を対象に抗がん剤と全身放射線照射にて体内リンパ球除去前処置後，体外で大量培養した腫瘍浸潤リンパ球(TIL)を体内へ戻す TIL 養子免疫細胞療法によって，奏効率 70%という驚異的な結果を報告している[4]．この治療方法は，GMP 準拠の cell processing center 設備や専門的な培養技術などを要するため，どの施設でもできる治療方法ではない．抗体療法は，B 細胞リンパ腫に対する rituximab，乳がんに対する trastzumab などすでに広く臨床で使用されており，抗原特異的受動免疫療法に分類される．

国内では，抗原特異的能動免疫療法の中でもペプチドワクチン療法が精力的に研究が行われており，ペプチドワクチンの早期創薬化を目的としたプロジェクトが先端医療開発特区(スーパー特区)で採択された．以下では，がん免疫療法のなかでも実地臨床において比較的施行しやすいと思われるワクチン療法，抗原感作樹状細胞療法の国内外の臨床試験の現状について述べる．

2．がん種別のワクチン開発状況について(表 2)

1）肺がん

肺がんでは，GlaxoSmithKline (GSK) 社が，MAGE-A3 蛋白ワクチン「MAGE-A3 ASCI」の臨床試験を進めている．Vansteenkiste らは，非小細胞肺がん完全切除後の再発予防目的に，MAGE-A3 蛋白ワクチン療法無作為化二重盲検比較第 2 相臨床試験の結果，術後再発率が 27%軽減したと報告した[5]．

表 2　がん種別ワクチン開発状況

対象がん種	開発名，抗原名	投与内容	海外/国内	開発段階
肺がん	MAGE-A3 ASCI	蛋白	海外	Phase Ⅲ
肺がん	WT1	ペプチド	国内	Phase Ⅰ
脳腫瘍	DCvax-brain	自己由来腫瘍細胞抽出抗原感作した自己樹状細胞	海外	スイスで承認
脳腫瘍	WT1	ペプチド	国内	Phase Ⅱ
脳腫瘍	テーラーメイドペプチドワクチン	ペプチド	国内	Phase Ⅰ
脳腫瘍	自家ワクチン	自己由来ホルマリン固定腫瘍	国内	Pilot study
腎細胞がん	Oncophage	自己由来腫瘍細胞抽出熱ショック蛋白 gp96	海外	ロシアで承認
腎細胞がん	CA9	ペプチド	国内	Phase Ⅱ
前立腺がん	Provenge	前立腺がん抗原 PAP/GM-CSF 融合蛋白感作した自己樹状細胞	海外	Phase Ⅲ
前立腺がん	テーラーメイドペプチドワクチン	ペプチド	国内	Phase Ⅰ/Ⅱ
膵がん	GV1001	ペプチド	海外	Phase Ⅲ：有効示せず
膵がん	VEGFR2	ペプチド	国内	Phase Ⅰ
肝細胞がん	GPC3	ペプチド	国内	Phase Ⅰ
子宮頸がん	GARDASIL	遺伝子組み換えウイルス様粒子	海外	諸外国で承認
子宮頸がん	Cervarix	遺伝子組み換えウイルス様粒子	海外	諸外国で承認

またその後の追跡調査の結果，「MAGE-A3 ASCI」投与群にみられた有害事象は，注射から 24 時間以内の注射部位の軽度の反応と発熱程度で，化学療法を行った場合と比べると非常に軽かった．この結果より「MAGE-A3 ASCI」の術後の再発リスク減少作用は化学療法と同程度で，副作用は化学療法に比べ低いことを示した[6]．現在大規模な第 3 相臨床試験が進行中である．

国内では，岡らが，肺がん症例 10 例に対して WT1(Wilms' tumor)ペプチドワクチン療法第 1 相試験の結果，3 例の腫瘍マーカー低下例を認めたと報告しているが[7]，国内では肺がんに対するがんワクチン療法の開発が十分進んでいるとはいえない．

2）脳腫瘍

Northwest Biotherapeutics 社は，自己由来の腫瘍細胞抽出抗原にて刺激した自己由来樹状細胞をワクチンする「DCvax-brain」をがん治療ワクチンとして初めて開発し，2007 年スイスで脳腫瘍を対象に承認された．Glioblastoma multiform を対象とした第 1/2 相試験後の長期追跡調査の結果が今年の 2 月に発表され，手術，放射線療法，化学療法からなる標準治療のみを受けた患者の 5 年生存率は，5%未満であるのに対し，DCvax-brain ワクチン群では，5 生率 25%，2 生率 68%と非常に良好な結果だったと報告した．現在，多施設による大規模第 2 相臨床試験が進行中である．

国内では，Izumoto らが，HLA-A24 陽性 glioblastoma multiform を対象にした WT1 ペプチドワクチン療法の第 2 相臨床試験の結果，21 例中，2 例部分奏効 PR，10 例安定 SD と報告している[8]．Yajima らは，進行悪性グリオーマに対して患者個々に免疫反応のあった 4 エピトープペプチドを選択しワクチンする，テーラーメイド型ペプチドワクチン療法第 1 相臨床試験 21 症例において，5 例部分奏効 PR，8 例安定 SD と良好な成績を示した[9]．Ishikawa らは，glioblastoma multiform を対象に自己由来ホルマリン固定腫瘍ワクチン療法のパイロットスタディを行った結果，12 症例中，1 例に完全奏効 CR，1 例部分奏効 PR，2 例 MR，1 例安定 SD，7 例増悪 PD との結果を報告している[10]．

3）腎細胞がん

腎細胞がんでは，Antigenics 社が患者自己由来腫瘍細胞から熱ショック蛋白である gp96 を抽出精製し，投与するワクチン「Onchophage」を開発した．gp96 などの熱ショック蛋白質はシャペロン分子としてがん抗原由来の多種類のペプチドと結合した形で存在しており，自己腫瘍由来 gp96 の抽出精製は同時に，自己腫瘍由来の多種がん抗原ペプチドも精製され，ワクチンされることになる．第 3 相臨床試験の結果，ワクチン群とコントロール群に全生存期間に差はみられなかったが，無再発生存期間では，ワクチン群で 45%改善がみられた．ロシア，EU ですでに 2008 年に承認されている．

国内では，Uemura らが，HLA-A24 陽性進行腎細胞がん（明細胞がん）を対象に，IFN-α 併用 CA9 (carbonic anhydrase 9) ペプチドワクチンの第 2 相臨床試験について本年の ASCO で報告した[11]．ワクチン症例 21 例中，肺，リンパ節転移を有した 1 例で，完全奏効 CR となり，その後 16 カ月再発がみられなかった．また，多発肺転移を有した 1 例が部分奏効 PR となった．他 3 例安定 SD という，良好な結果を示した．

4）前立腺がん

前立腺がんでは，Dendreon 社が前立腺がん抗原 PAP (prostatic acid phosphatase) と GM-CSF との融合蛋白で患者由来樹状細胞を刺激し，投与するワクチン「Provenge」を開発した．転移性無症候性のホルモン抵抗性前立腺がんに対して，プラセボ対照無作為二重盲検第 3 相臨床試験が行われ，無増悪生存期間に統計学的有意差はみられなかったが，全生存期間では有意差がみられ，ワクチン投与群で，生存期間中央値が 4.5 カ月延長した[12]．

国内では，Noguchi らが，ホルモン抵抗性前立腺がんを対象に，14 種類の peptide のうち，vaccine 前血漿中に抗体が存在する 4 epitope peptide を患者個々に合わせて選択し，4 peptides 同時ワクチンする personalized peptide vaccine (PPV) を試行した結果を ASCO2009 にて報告した[13]．low dose EMP (280 mg/day) + PPV 群と full doze EMP (560 mg/ml) のみの群で無作為な臨床試験が行われた結果，low dose EMP + PPV 群での無増悪生存期間が 246 日間であるのに対し，full dose EMP のみでは 85 日間であり，PPV 群で優位な延長を認めた．

5）膵がん

Pharmexia 社は，ヒトテロメラーゼ逆転写酵素由来の class Ⅱエピトープペプチドワクチン〔hTERT (611-626)〕，「GV1001」を開発した．進行膵がんを対照に，gemcitabine 併用 GV1001 ワクチン治療無作為比較第 3 相臨床試験の結果が，ASCO2009 で報告されたが，GV1001 ワクチン群での有効性を示せなかった[14]．

国内では，Yamaue らが，HLA-A24 陽性進行膵がんを対象に，gemcitabine 併用 VEGFR2 ペプチドワクチンの第 1 相臨床試験の結果を，ASCO2009 で報告した[15]．エントリーされた 18 症例中，1 例部分奏効 PR，11 例安定 SD で，disease control rate が，67% (12/18 症例) で，生存期間中央値が 8.7 カ月だった．現在，オンコセラピー・サイエンス社による大規模な無作為第 2 相，第 3 相臨床治験が進行中である．

6）肝細胞がん

肝細胞がんにおいては，海外では目立った成績を示すがんワクチンは開発されていない．国内では，我々が，HLA-A24，A2 陽性進行肝細胞がんを対象に，Glypican-3 (GPC3) ペプチドワクチンの第 1 相臨床試験を実施している．安全性に問題はなく，ほぼ全例に末梢血中ペプチド特異的 CTL の頻度の増加が検出され，その頻度には投与量依存性が示唆された．ワクチン後のがん組織内に，CD8 陽性キラー T 細胞が多数浸潤していることが，複数の症例で証明できた．約 60%の症例において初回ワクチン投与後 2 カ月の間に腫瘍マーカー PIVKA-Ⅱの低下を認め，RECIST 基準での評価では約 60%の症例が SD であった．30 mg，3 回投与の 1 例に腫瘍の縮小や消失などの著明な臨床効果（部分奏効 PR）が出現した．今後はもう他に治療法がない進行肝細胞がん患者にとって有用であるかを第 2 相試験で検証するとともに，このようなワクチン療法は元来，腫瘍がない，あるいは CT でみえない腫瘍があったと

しても腫瘍量が少ない状態でこそ威力を発揮すると考えられ，手術やRFAなどの肝細胞がん根治的治療後の再発予防の第2相試験を実施する．GPC3は，肝細胞がんだけでなく，悪性黒色腫，小児がん(肝芽腫，神経芽腫，腎芽腫)，卵巣明細胞がん，卵黄嚢腫瘍，絨毛がん，肺扁平上皮がんにも発現しており，それらのがん種に対しての応用も期待される．

7）子宮頸がん

以上，がん治療用ワクチンを述べてきたが，がん予防用ワクチンも存在する．子宮頸がんの原因である，ヒトパピローマウイルス(HPV)感染を予防するワクチンとして，米Merck社「GARDASIL」と英GlaxoSmithKline社「Cervarix」が開発され，2006年に米国をはじめ諸外国で承認された．このワクチンの登場により発がん性HPVである16型と18型の感染を予防し，約70%の子宮頸がんが予防可能となった．日本では現在申請中である．

結語

現在のところ，がんワクチン療法としては，抗がん剤を凌駕する腫瘍縮小効果を示せていないのが現状ではあるが，重篤な毒性がほぼないということと，抗がん剤とは異なった機序による抗腫瘍効果を示し，がん種によっては延命効果もみられることから，抗がん剤や分子標的治療薬との併用による相乗効果や，抗がん剤治療に耐えられない患者への適応など，新たながん治療の選択肢の一つに十分なりうる．またワクチンは本来ウイルス感染予防治療として発達してきたが，がんワクチンも今後臨床試験が蓄積されていけば，がん予防にも威力を発揮することが期待されている．免疫学は，まだまだ未知の領域であり，今後この領域からがん治療に対するブレイクスルーを期待したい．

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Glypican-3 is a useful diagnostic marker for a component of hepatocellular carcinoma in human liver cancer

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Received September 10, 2008; Accepted October 27, 2008

DOI: 10.3892/ijo_00000190

Abstract. Primary liver cancers are classified into three types based on their morphology and cytogenetic characteristics hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and combined hepatocellular and cholangiocarcinoma (CHC). It is often difficult to distinguish these liver tumors. Glypican-3 (GPC3) is serological and histochemical marker of hepatocellular carcinoma. In order to separate these three types of liver cancers, we analyzed the GPC3 expression in 85 liver resection specimens, including 46 HCCs, 28 ICCs and 11 CHCs. GPC3 immunohistochemical staining was used to distinguish HCC from ICC by comparing with the conventional biomarker, α-fetoprotein (AFP). The immunostaining of GPC3 was identified in 78.3% (36/46) of HCCs, 60% (9/15) of well differentiated, 88.9% (16/18) of moderately differentiated and 84.6% (11/13) of poorly differentiated HCCs. It was negative in the ICCs. We confirmed that GPC3 expression is specific to HCC component (8/11, 72.7%) but few samples also showed weakly in ICC component (2/11, 18.2%) of CHC sections among 11 cases compared with HCC biomarkers including AFP and hepatocyto paraffin 1 (HepPar1), and ICC biomarkers cytokeratin (CK) 7 and CK19. Three cases in which the macroscopic features resembled ICC did not express GPC3 even in the pathological HCC component. Most (10/11, 91%) of the pathological cholangiocarcinoma components in CHC showed positive staining for CK7 and CK19. The results of this study suggest that GPC3 is a biomarker that is sensitive and specific to HCC component of CHC, and CK7 and CK19 are markers for pathological cholangiocarcinoma component of CHC.

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Abbreviations: HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; CHC, combined hepatocellular and chorangiocarcinoma; GPC3, glypican-3; AFP, α-fetoprotein; HepPar1, hepatocyto paraffin 1; CK, cytokeratin; CC, cholangiocarcinoma; cp, component

Key words: hepatocellular carcinoma, intrahepatic cholangiocarcinoma, combined hepatocellular and chorangiocarcinoma, glypican-3, CK7, CK19, immunohistochemical analysis

Introduction

Liver cancer is one of the common malignancies that are rapidly increasing throughout the world. Primary liver cancers are classified into three types based on their morphology and cytogenetic characteristics, hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and combined hepatocellular and cholangiocarcinoma (CHC). HCC is hepatocyto-origin, and ICC is from the epithelium of the intrahepatic bile duct. CHC is a rare type of liver cancer with features of both hepatocellular and biliary differentiation (1-3). The pathological structure of CHC is composed of hepatocellular element showing bile production, an intercellular bile canaliculi or trabecular growth pattern and cholangiocellular component showing mucin production or gland formation.

Because of their rapid growth rate and the lack of accurate ways of diagnosis in the early stages, the prognosis and the survival rate for liver cancer patients remain poor. Currently, ultrasound sonography (US), computed tomography (CT), magnetic resonance imaging (MRI), and histopathological examination for tumor biopsy are used for diagnosis. However, distinguishing the three different primary liver tumors is often a challenging task in diagnosis, for which immunohistochemical analysis for specific antigens is a helpful tool: α-fetoprotein (AFP) and hepatocyto paraffin 1 (HepPar1) for HCC (4-8) and cytokeratin (CK) 7 and CK19 for ICC (9-11).

Glypican-3 (GPC3) was discovered as a potential serological and histochemical marker whose expression is specific for HCC (12-16). GPC3 belongs to glypican family that is a group of heparan sulfate proteoglycans linked to the outer surface of cell membrane through a glycosylphosphatidylinositol anchor (17). In mammals, six members of GPCs have been reported, GPC1 to GPC6. GPCs are released from the cell surface by a lipase called Notum to regulate the signaling of Wnts, Hedgehogs, fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) (18-25). Depending on the cellular context, their function can be stimulatory or inhibitory activity, or signaling. The expression of GPC3 is detected in placenta and fetal liver, but not in other normal organs. During hepatic carcinogenesis, GPC3 have been reported to reappear in HCC and to be released into serum (12,13,15,26). Its expression is also detected in melanoma (27-29). The functions of GPC3 in cancer cells are still unclear.

In this study, we examined whether immunohistochemical analysis for GPC3 can be used to distinguish HCC from ICC, if so, how effectively GPC3 can be detected, compared to other biomarkers that are conventionally used. We demonstrate that distinguishing HCC from ICC by detecting the expression of GPC3 enables more accurate diagnosis.

Materials and methods

Case selection. We selected 85 cases of liver tumors from the surgical pathology files from 1992 to 2006 of National Cancer Center Hospital East, Kashiwa, Chiba, Japan. The cases included 46 primary HCCs, 28 ICCs, and 11 CHCs that underwent hepatectomy. All identifiers were eliminated to protect patients' identities. Size of the tumor and any clinicopathologic factors (age, sex and grade of tumor) were matched between HCC and ICC. The 46 cases of HCCs occurred in 33 men and 13 women with a mean of age at 65.3 years (range, 44-80 years). HCC was subclassified into well (n=15), moderately (n=18), and poorly (n=13) differentiated types according to the World Health Organization classification criteria. The 28 cases of ICC consisted of 18 men and 10 women. Their mean age was 65.7 years (range, 51-82 years). All 28 resected cases of ICC were confirmed by hematoxylin-eosin (H.E.) staining.

The 11 cases of CHC included 7 men and 4 women with a mean age of 62.5 years (range, 47-76 years). All CHCs were pathologically confirmed after surgery.

Tissue samples. Liver tissue sections were retrieved from the files of the Department of Pathology in our institution. All liver specimens were prepared from surgically resected tumors and adjacent parenchyma. They were fixed in 10% formalin and paraffinized for routine histological examination.

Immunohistochemical staining procedure. Six-micrometer-thick sections were made from the paraffin-embedded blocks. Subsequently the sections were deparaffinized in xylene and rehydrated through ethanol to water. Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 20 min. For antigen retrieval, Sections were heated in 10 mM citrate buffer (pH 6.0) with microwave for 15 min in a water bath at 95°C. Only for CK7 immunostaining, sections were digested by Proteinase K (DakoCytomation, Carpenteria, CA) for 5 min at room temprature. Slides were then allowed to cool down. The prediluted primary antibodies, monoclonal anti-GPC3 (dilution 1:300, 1G12; Biomosaics, Inc., Burlington, VT), anti-AFP (dilution 1:400, DakoCytomation), anti-HepPar1 (dilution 1:100, DakoCytomation), anti-CK7 (dilution 1:100, DakoCytomation), and CK19 (dilution 1:200, DakoCytomation) were added to cover each slide, and the slides were incubated for 2 h at room temperature. Slides were washed 3 times in phosphate-buffered saline (PBS)/Tween for 5 min each. Mouse Envision Polymer (DakoCytomation) was used as a secondary antibody for 30 min at room temperature followed by washes in PBS/Tween 3 times for 5 min each. Diaminobenzidine chromagen (DakoCytomation) was added to each slide and incubated for 2 min. Slides were washed in distilled water, counterstained with hematoxylin and dehydrated in xylene. To analyze GPC3 expression, the immunohistochemical results were classified according to the number of positive cells as follows: -, negative (<10%); ±, weakly positive (10-30%); + positive (>30%). To validate the data in GPC3 as a marker for HCC, parallel staining for AFP of 46 cases were further analyzed. For 11 CHC cases, AFP, HepPar1, CK7 and CK19 were stained and compared with GPC3 staining pattern.

The slides were examined independently by 3 observers (Shirakawa H, Kuronuma T and Nakatsura T) and then collectively by 2 more pathologists (Hasebe T and Nakano M).

Statistical analysis. Differences in proportion were tested by the χ^2 test. Differences in the means of each subgroup were tested using the Student's t-test. P-value of <0.05 was considered statistically significant.

Results

GPC3 was present in 80% of HCC and negative in ICC. In order to examine the levels and pattern of GPC3 expression, 46 cases of HCC and 28 cases of ICC were immunohistochemically analyzed. GPC3 was detected in 36 cases (78%) of HCC (Fig. 1a), and no expression of GPC3 was found in any of the ICC patients (Fig. 1b). The GPC3 staining was diffused throughout (Fig. 1c) or localized in a granular pattern in the cytoplasm (Fig. 1d). In other cases, GPC3 was observed at the plasma membrane (Fig. 1e). Previously GPC3 is shown to bind to the cell membrane (16), however, those cases with membranous GPC3 had staining in the cytoplasm as well, but there was no case of GPC3 located only at the plasma membrane. When sensitivity of GPC3 was evaluated, 36 cases (78%) were positive for GPC3 when only 16 cases (35%; $P<0.0001$) were stained for AFP in HCC suggesting that GPC3 is more sensitive than AFP. Thus, GPC3 was confirmed to be specific and sensitive to HCC compared to AFP.

GPC3 expression increased in moderately and poorly differentiated HCC. In terms of GPC3 expression and tumor

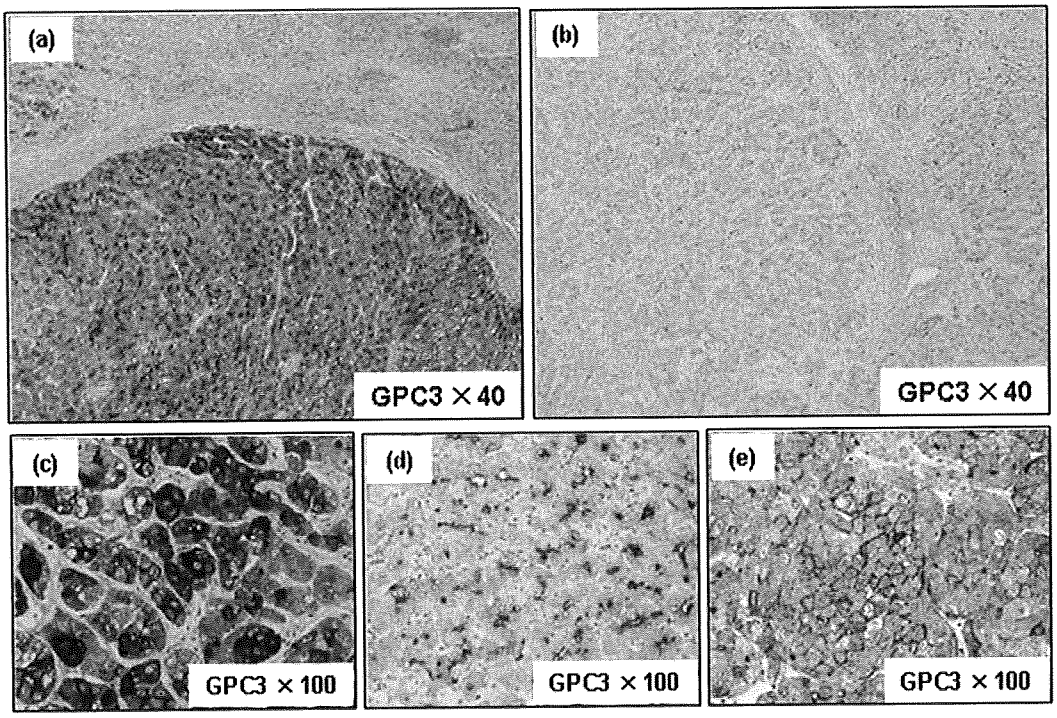

Figure 1. GPC3 expression was specific to HCC and absent in ICC. Immunohistochemical detection of GPC3 expression in HCC (a) and ICC (b) (magnification, x40). Immunostaining patterns of HCC: (c) diffuse in cytoplasm, gulanular in cytoplasm (d), and membranous (e).

Table I. Correlation of positive for GPC3 staining and tumor grade.

Grade of tumor	HCC					ICC		
		GPC3						
	No. of case	-	±	+	positivity	No. of case	GPC3 positivity	P-value
Well-differentiated	15	6	5	4	9 (60%)	8	0 (0%)	
Moderately differentiated	18	2	4	12	16 (89%)	10	0 (0%)	
Poorly differentiated	13	2	5	6	11 (85%)	10	0 (0%)	
Total	46				36 (78%)	28	0 (0%)	<0.0001

-, negative (<10%); ±, weakly positive (10-30%); +, positive (>30%).

differentiation level, GPC3 was expressed in 9 (60%) of 15 well differentiated, 16 (89%) of 18 moderately differentiated and in 11 (85%) of 13 poorly differentiated HCC (Table I). AFP was expressed in 3 (20%) of 15 well differentiated, 6 (33%) of 18 moderately differentiated and in 7 (54%) of 13 poorly differentiated HCC (data not shown). The expression level of GPC3 was lower in well differentiated HCC than in the other HCC grades, though the difference was not statistically significant (well- vs. moderately differentiated: P=0.054, well- vs. poorly differentiated: P=0.150). Thus, GPC3 expression is also a good indicator for malignancy levels.

GPC3 expression was observed specifically in pathological HCC component in CHC. There are discrepancies between preoperative diagnosis and pathological findings for CHC patients. Diagnostic results and the expression of tumor markers of 11 CHC patients are summarized in Table II. Initial diagnosis was carried out by H.E. staining. Among these 11 patients, 7 patients (63.6%) were diagnosed as HCC and 3 (27.3%) were ICC. Only 1 patient (9%) of the 11 CHC was correctly diagnosed as CHC. To seek the possibility to use GPC3 immunostaining to detect HCC component (cp) in CHC, combination of antibodies against GPC3, AFP, HepPar1, CK7 and CK17 were used. In addition to AFP, HepPar1 is frequently used as marker for HCC (4-8) and CK 7 and CK19 for ICC (9-11).

Among 11 CHC cases, 4 cases preoperatively diagnosed as HCC were chosen to represent the collision and transitional type of CHCs based on the macroscopic features

Table II. Correlation of immunostaining varieties and pathological components of CHC.

Pt. no.	Preoperative diagnosis	Macroscopic diagnosis	Pathological hepatocellular carcinoma component					Pathological cholangiocarcinoma component				
			GPC3	AFP	HepPar1	CK7	CK19	GPC3	AFP	HepPar1	CK7	CK19
1	HCC	CHC	+	+	-	+	+	-	-	-	-	-
2	HCC	HCC	+	-	-	-	-	-	-	+	+	+
3	HCC	HCC	+	-	+	-	-	±	-	-	+	+
4	CHC	HCC	+	+	+	-	-	±	-	-	+	+
5	HCC	CHC	+	-	+	-	-	-	-	-	+	+
6	HCC	CHC	+	-	-	-	-	-	-	+	+	+
7	ICC	CHC	±	-	-	±	+	-	-	-	+	+
8	HCC	HCC	+	+	-	-	-	-	+	-	+	+
	Total ±		8/8	3/8	3/8	3/8	2/8	2/8	1/8	2/8	7/8	7/8
	positive rate (%)		100	38	38	38	25	25	13	25	88	88
9	ICC	ICC	-	-	-	-	-	-	-	-	+	+
10	HCC	ICC	-	-	-	+	±	-	-	-	+	+
11	ICC	ICC	-	-	-	+	+	-	-	-	+	+
	Total ±		0/3	0/3	0/3	2/3	2/3	0/3	0/3	0/3	3/3	3/3
	positive rate (%)		0	0	0	67	67	0	0	0	100	100

-, negative (<10%); ±, weakly positive (10-30%); +, positive (>30%); HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; CHC, combined hepatocellular and cholangiocarcinoma; GPC3, glypican-3; AFP, α-fetoprotein; HepPar1, hepatocyto-paraffin 1; CK, cytokeratin; CC, cholangiocarcinoma.

in cut surface. In Fig. 2, macroscopic observation and the immunostained histological sections are shown. These sections include 2 elements with pathological HCC cp forming bile production and trabecular growth pattern by eosinophilic staining and cholangiocarcinoma (CC) cp forming mucin production or gland formation by basophilic staining. Cases 1-8 were GPC3 positive, and cases 9-11 were negative for GPC3 in the HCC cp. Macroscopic, histological and immunohistochemical features of cases 2, 6, 8 and 10 are shown in Fig. 2a, b, c and d. Case 2 had greenish white and yellow nodules within the same tumor mass in the cut surface. HCC subtypes such as simple nodular and confluent multinodular type exist. Case 2 exhibited the features of HCC with multinodular type (Fig. 2a-i). Pathological diagnosis by H.E. staining revealed CHC pathologically (Fig. 2a-ii and -iii), which was so-called 'collision'-type tumor as reported by Goodman *et al* (30). A 'collision'-type tumor is coincidental occurrence of HCC and CC within the same tumor mass (31). GPC3 was positive (Fig. 2a-iv), but AFP and HepPar1 were not detected in HCC cp (Fig. 2a-v and -vi). Although HepPar1 is generally used as HCC marker, it was unexpectedly stained in CC region as well as CK7 and CK19 (Fig. 2a-vii and -viii).

Case 6 showed pale and lobulated phenotype in the cut surface macroscopically (Fig. 2b-i), and pathological diagnosis was also confirmed by H.E. staining (Fig. 2b-ii and -iii). This was so-called 'transitional' type tumor (30). A 'transitional' type tumor has an area of HCC that appears to transform into CC (31). GPC3 was stained in pathological HCC cp (Fig. 2b-iv) where AFP was negative (Fig. 2b-v). The HCC region was surrounded by pathological CC cp with the staining for CK7 (Fig. 2b-vii). HepPar1 and CK19 were detected in the same region with CC cp (Fig. 2b-vi and -viii). HepPar1 stained the CC cp as in case 2. The immunoreactivity of CK19 was not consistent with that of CK7.

Case 8 was diagnosed as HCC similarly to cases 2 and 6, but mixed tumor masses with white and gray in the cut surface were observed (Fig. 2c-i and c-ii). Both GPC3 and AFP were positive in HCC cp (Fig. 2c-iv and -v). HepPar1 was stained in CC cp (Fig. 2c-vi). CK7 and CK19 were positive in CC cp (Fig. 2c-vii and -viii), especially CK19 was more specific for CC cp than CK7. These three cases (cases 2, 6 and 8) indicated that detecting GPC3 can compensate for AFP and enhance the ability to identify the presence of HCC cp in CHC.

Cases 9, 10 and 11 were negative for GPC3 expression in several tumors. Macroscopically, they had the features of ICC with irregular shaped, white solid tumor masses. As an example, case 10 is shown in Fig. 2d. Althogh case 10 was diagnosed as HCC preoperativerly, it showed macroscopic freatures of ICC with the presence of abundant fibrous stroma and indistinct tumor margin (Fig. 2d-i). This case was later diagnosed as CHC based on the pathological examination (Fig. 2d-ii and d-iii). GPC3, AFP and HepPar1 were not detected in either HCC cp or CC cp (Fig. 2d-iv, -v, and -vi). CK7 was stained diffusely in the tumor (Fig. 2d-vii), and CK19 expression was more specific in CC cp than CK7 (Fig. 2d-viii). These 3 cases showed positive staining

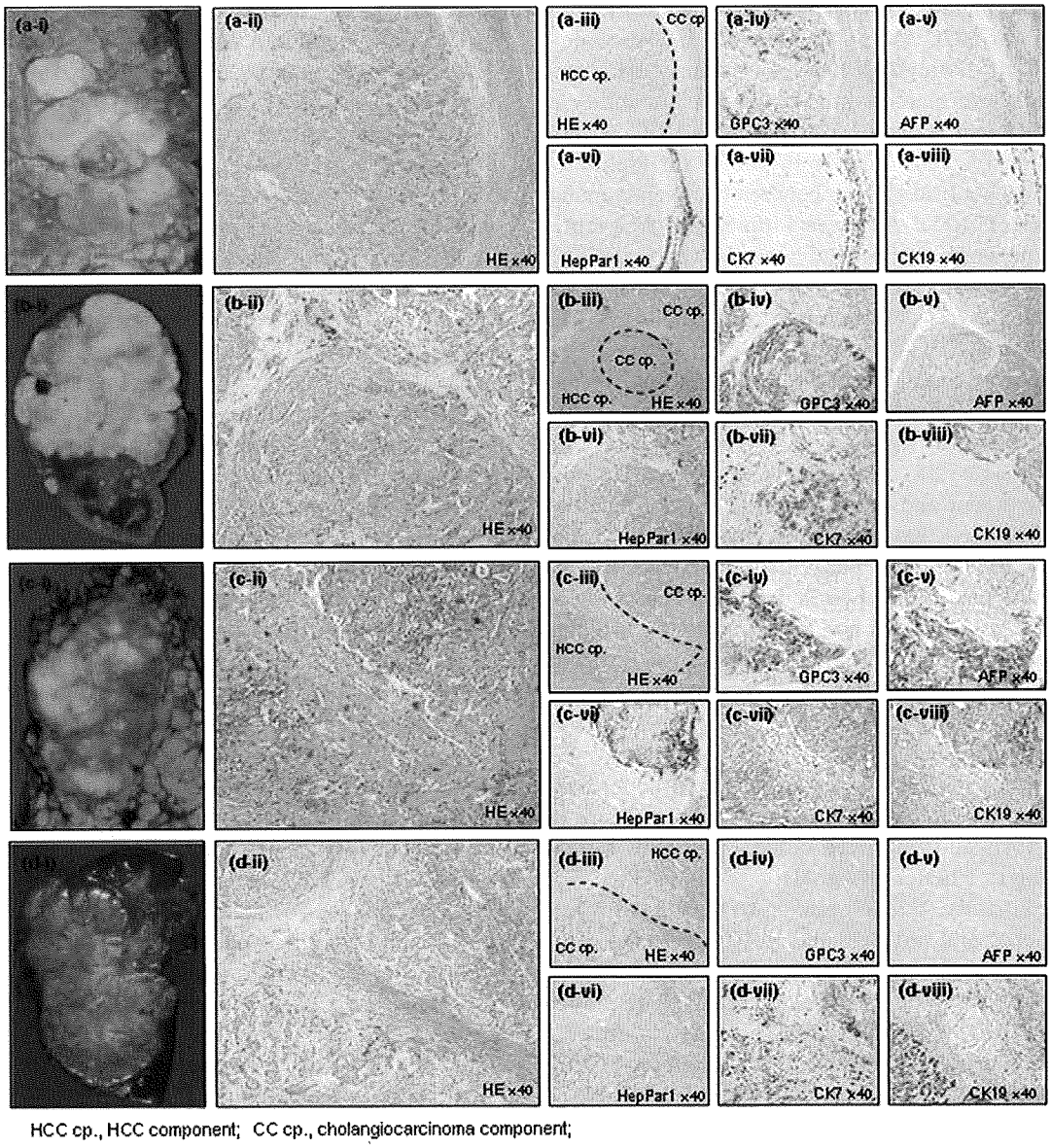

Figure 2. Macroscopic, histological and immunohistochemical features of four cases of CHC, a, case 2; b, case 6; c, case 8; d, case 10 in Table II. (a-i) Macroscopic feature in cut surface of case 2 tumor. (a-ii) The histological structure can be also devided into 2 types. HCC component showed expansive growth oppressing the cholangiocarcinoma component. (a-iii) Collision border between hepatocellular carcinoma and cholangiocarcinoma component are indicated as dots. The tumor cells within mainly hepatocellular carcinoma component showed only expression of GPC3 (a-iv) without expression of AFP (a-v). In the opposite side, the glandular area with cholangiocarcinoma component shows HepPar1 (a-vi), CK7 (a-vii) and CK19 expression (a-viii). (b-i) Case 6 shows macroscopic CHC feature in tumor cut surface that was suspected out HCC preoperatively. (b-ii) The histological cholangiocarcinoma component forming trabeculae with columnar appearance was surrounded by HCC component forming hepatoid structure. (b-iii) A dotted line is a boundary of HCC in the H.E. staining. The tumor cells within transitional region were positive for GPC3 (b-iv), CK 7 (b-vii) and CK 19 (b-viii). The difference was recognized between hepatocellular carcinoma component and cholangiocarcinoma component because GPC3 positive area encircled the CK7 area. The expressions of AFP (b-v) and HepPar1 (b-vi) were not observed. (c-i) Though case 8 was also suspected to be HCC preoperatively, the macroscopic features showed atypical HCC with mixed white and gray and indistinct tumor border. (c-ii) The cholangiocarcinoma component was obviously composed of structural gland formation. (c-iii) Collision area was distinguished histopathologically by a dotted line. The tumor cells of HCC component showed not only GPC3 (c-iv) but also AFP expression (c-v). In the glandular area of cholangiocarcinoma component, HepPar1 was expressed (c-vi), but CK7 not at all (c-vii) and CK19 shows weak positive expression (c-viii). (d-i) Case 10 shows macroscopic ICC features in tumor cut surface that was suspected as HCC preoperatively. (d-ii) The histological structure can be devided into 2 types with cholangiocarcinoma component forming trabeculae with columnar appearance and HCC component forming hepatocellular structures. (d-iii) A dotted line is a boundary of HCC in the H.E. GPC3 (d-iv), AFP (d-v) and HepPar1 (d-vi) were not stained, but CK7 (d-vii) and CK19 (d-viii) stained the cholagiocarcinoma component.

for CK7 and CK19 in CC cp, but not AFP or HepPar1 in HCC cp. Therefore, accuracy of CHC diagnosis can be achieved by combination of multiple tumor markers in addition to morphological characteristics: GPC3 that is specific for pathological HCC cp of CHC, and CK7 and CK19 that are specific for pathological CC cp of CHC.

Discussion

The diagnosis for HCC, ICC and CHC has been routinely performed by histopathological examination. Additionally, diagnosis of HCC is done by supplementary immunohistochemical analysis for AFP and HepPar1. Until now, though

the sensitivity is limited, AFP has been regarded as the most useful marker for HCC (4,32-34). HepPar1 is also widely used for HCC to distinguish between primary HCC and ICC. However, both markers are limited for the ability to discriminate different levels of malignancy in HCC because its sensitivity drops substantially in poorly differentiated HCC, and it does not discriminate between benign and malignant liver cancers (35). As these biomarkers frequently results in misdiagnosis, in this study, we showed that GPC3 is more sensitive to detect HCC compared to AFP. Due to the fact that GPC3 was downregulated in ICC (36), GPC3 may help to separate HCC from ICC.

CHC is the least common primary cancer of the liver but followed by an aggressive growth, it tends to metastasize to many organs leading to significantly poorer prognosis than HCC and ICC (31,37,38). Correct diagnosis leads to both appropriate treatment and better outcome for the patients. Nishie, *et al* reported that one third (nine of 27 cases) of patients with CHC were correctly diagnosed by enhanced computed tomography (39). In our study, only one of the 11 (9.1%) patients with CHC was correctly diagnosed before operation without fine needle aspiration biopsy. The difficulty to pathologically distinguish CHC from HCC and ICC comes from glandular or pseudoglandular structures in HCC and solid or trabecular patterns in CC (37,38). We believe that combination with histopathological examination with GPC3 immunostaining and radiological examination can bring an accurate diagnosis and improved clinical therapies for the patients leading to a better prognosis.

We showed that the immunostaining for GPC3 is specific for HCC patients and not detected in ICC patients. This confirmed that detecting GPC3 may improve the method to diagnose CHC. Of the 11 cases of CHC, 8 displayed GPC3 expression in restricted area of HCC cp. We demonstarated that immunohistochemical staining of GPC3 in liver tumor helps to recognize the pathological HCC cp more precisely. GPC3 expression was observed with high frequency in the HCC cp compared with AFP and HepPar1. HepPar1 was unexpectedly stained in CC cp, but this has been observed previously as well (7,40). This could be due to a transition from HCC to ICC where HepPar1 is one of the molecules that is downregulated at later stages in the process. CK7 and CK19 have been already reported as good markers of biliary epithelial differentiation (41). These were highly expressed in pathological CC cp (10/11, 91%) in CHC. The positive immunoreactivity of CK19 was more distinct than that of CK7 whose staining was weaker. Our immunohistochemical data disclosed that GPC3 can be a better marker specific for HCC leading to a better confirmation for HCC component of CHC as well as for HCC. Moreover, it provided evidence of the biologic behavior of such combined tumors, which are phenotypically and genetically leaning toward either ICC with predominant biliary differentiation or HCC with hepatocellular differentiation (42,43).

Employing multiple tumor markers may also allow the accurate diagnosis of CHC containing both hepatocellular and biliary differentiation. Concerning sensitivity and specificity, the combination of GPC3 for HCC cp and CK19 for ICC cp seems to be useful in the diagnosis of liver cancer. For CHC, GPC3 positive/CK19 negative profile suggests HCC, GPC3 positive/CK19 positive indicates CHC, and GPC3 negative/CK19 positive essentially rules out HCC and suggests the possibility of CC or CHC.

We developed a new anti-cancer immunotherapy with GPC3 as a target (44-47), and the phase I clinical trial of GPC3-derived peptide vaccination for advanced HCC is now on going. Because this new immunotherapy is not indicated for ICC, immunohistochemical staining of GPC3 is a useful method to select eligible patients. Furthermore, if CHC would be justified as a target of our immunotherapy in future, immunohistochemical analysis for GPC3 expression is indispensable for the process of patient selection.

GPC3 is expressed in the group of cells that are AFP-positive and/or CK7/19-positive in injured livers with activation of oval cell compartment; an indication for liver repair and regeneration (48). In addition, CK7, CK19 and AFP are frequently expressed in biliary epithelial cells (49,50) and in immature fetal hepatoblasts (51,52). Liver progenitor cells originate from the canal of Hering, lined by both hepatocytes and biliary ductular epithelial cells (53). It is not clear whether GPC3 is expressed in hepatic embryonic progenitor cells or cancer stem cells, but GPC3 may be a marker for hepatic progenitor/stem cells. In CHC cases of 2, 3 and 4, GPC3, CK7 and CK19 coincided in the regions of HCC and CC. Although HCC and ICC are two different kinds of primary liver malignancies arising from different cell types as hepatocytes and cholangiocytes, co-localization of GPC3 and CK7/19 suggest that the CHC is originated from progenitor or oval cell. In addition, case 6 showed an HCC lesion with GPC3 positive immunostaining surrounded by CC (Fig. 2b). This finding suggests that GPC3-positive HCC tumor cells are derived from GPC3-negative CC mass. Moreover, we predict from the fact that GPC3 is expressed in embryonic liver and downregulated after birth in normal liver but reappears in cancer is due to its regulatory role in proliferative and dedifferentiated cells, like cancer cells that acquired a progenitor- or cancer stem cell-like characteristics.

In summary, we confirmed that GPC3 is a marker sensitive and specific for HCC, but not ICC. Moreover, we revealed that GPC3 was expressed specifically in the HCC cp in the CHC. Therefore, GPC3 is a molecule that is significant not only in clinical but also biological field. It is clinically an important biomarker that can be used for accurate diagnosis leading to a better treatment and prognosis. Also, biologically, it may be an indicator for the identity and the origin of the cancer cells.

Acknowledgments

This study was supported in part by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labor, and Welfare, Japan, and a grant-in-aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare, Japan. Foundation for Promotion of Cancer Research in Japan, Japan Research Foundation for Clinical Pharmacology and Research Resident Fellowship from the Foundation for Promotion of Cancer Research, Japan (H.S.). We thank Dr Chinatsu Kojima (Section for Cancer Immunotherapy, Investigative Treatment Division,

Research Center for Innovative Oncology, National Cancer Center Hospital East) for technical assistance.

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Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma

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(Received November 21, 2008/Revised April 10, 2009/Accepted April 19, 2009/Online publication June 2, 2009)

The relationship between overexpression of glypican (GPC)-3 that is specific for hepatocellular carcinoma (HCC) and the prognosis has not yet been clarified. We attempted to determine the expression profile of GPC3 in association with the clinicopathological factors by immunohistochemical analysis in HCC patients and investigated the potential prognostic value of GPC3 by comparing the survival rate between the GPC3-positive and GPC3-negative HCC patients. Primary HCC tissue samples (n = 107) obtained from patients who had undergone hepatectomy between 2000 and 2001 were analyzed. GPC3 expression was less frequently observed in well-differentiated HCC than in moderately and poorly differentiated HCC, the difference in the frequency being statistically significant. GPC3-positive HCC patients had a significantly lower 5-year survival rate than the GPC3-negative HCC patients (54.5 vs 87.7%, P = 0.031). Among 80 of the 107 (74.6%) patients with initial treatment who underwent hepatectomy, none of GPC3-negative HCC patients (n = 16, 20.0%) died during the follow-up period. No deaths were noted in the GPC3-negative HCC patients among the 71 (88.7%) patients with moderately and poorly differentiated HCC. Multivariate analysis identified GPC3 expression (P = 0.034) as an independent prognostic factor for the overall survival. We showed that GPC3 expression is correlated with a poor prognosis in HCC patients. (*Cancer Sci* 2009; 100: 1403–1407)

Hepatocellular carcinoma (HCC) is one of the most common malignancies and is ranked as the third most common cause of cancer-related death worldwide. HCC is generally associated with a poor prognosis, the 5-year survival rate after surgery has been reported to be as low as 25–39%, and systemic therapy with cytotoxic agents provides only marginal benefit.[1] Even in those patients in whom the tumor has been successfully removed, the 2-year recurrence rate can be as high as 50%.[2,3] Several clinicopathological factors including poor levels of differentiation of the cancer cells, large size of the tumor, portal venous invasion, and intrahepatic metastasis have been shown to contribute to the poor prognosis in patients of HCC. Despite the critical need for better methods for the diagnosis and treatment of HCC, the mechanisms underlying the development of HCC remain unclear.

Glypican (GPC)-3 was discovered as a potential serological and histochemical marker that is specific for HCC. GPC3 is a member of the glypican family and belongs to a group of heparan sulfate proteoglycans bound to the outer surface of the cell membrane through a glycosylphosphatidylinositol anchor.[4] In mammals, this family comprises six members, GPC1 to GPC6. GPC are released from the cell surface by a lipase called Notum to regulate the signaling of Wnts, Hedgehogs, fibroblast growth factors, and bone morphogenetic proteins.[5–9] Depending on the context, their functions exerted may either be stimulatory or inhibitory through these pathways. GPC3 has been detected in the placenta and fetal liver, but not in other adult organs. During hepatic carcinogenesis, GPC3 appears in the HCC tissue and is released into the serum.[10–12] In addition, its expression has also been reported in melanoma.[13–15]

A dramatic elevation of GPC3 expression has been reported in a large proportion of HCC, as determined by cDNA microarray analysis, whereas its expression has been shown to be less frequent in preneoplastic or entirely absent in non-neoplastic liver tissue.[16–18] This has led to the notion that GPC3 may have diagnostic usefulness as a marker of differentiation or a specific tumor marker in the case of HCC. However, until now, the relationship between GPC3 overexpression and the prognosis of HCC has not been clarified.

In the present study, we attempted to determine the tumor expression profile of GPC3 in association with clinicopathological factors in HCC patients by immunohistochemical analysis. We also investigated the potential prognostic value of GPC3 by analyzing the survival rate of GPC3-positive and GPC3-negative HCC patients. By elucidating the association between the GPC3 expression level in HCC tumors and the survival rate of the patients, we concluded that the GPC3 expression level is correlated with a poor prognosis in HCC patients.

Materials and Methods

Patients and tumor tissue samples. Primary HCC tissue samples (n = 107) were obtained from patients who underwent hepatectomy at the National Cancer Center Hospital East between 2000 and 2001. The histological types were assigned according to the criteria of the World Health Organization classification. Liver tissue sections prepared from the surgically resected tumors and adjacent parenchyma fixed in 10% formalin and embedded in paraffin were retrieved from the files of the Department of Pathology at our institution.

Immunohistochemical staining. Sections 6 μm thick were prepared from the paraffin-embedded blocks. The sections were deparaffinized in xylene and rehydrated through ethanol to water. Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 20 min. For antigen retrieval, sections were heated in 10 mM citrate buffer (pH 6.0) with microwave at 95°C for 15 min. The slides were then allowed to cool down, and the prediluted primary monoclonal anti-GPC3 antibody (dilution 1 : 300; Biomosaics, Burlington, VT, USA) was added to cover each slide, and the slides were incubated for 2 h at room temperature. Thereafter, the slides were washed three times in TBS–Tween 20 for 5 min each. Mouse Envision Polymer-horseradish

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doi: 10.1111/j.1349-7006.2009.01206.x

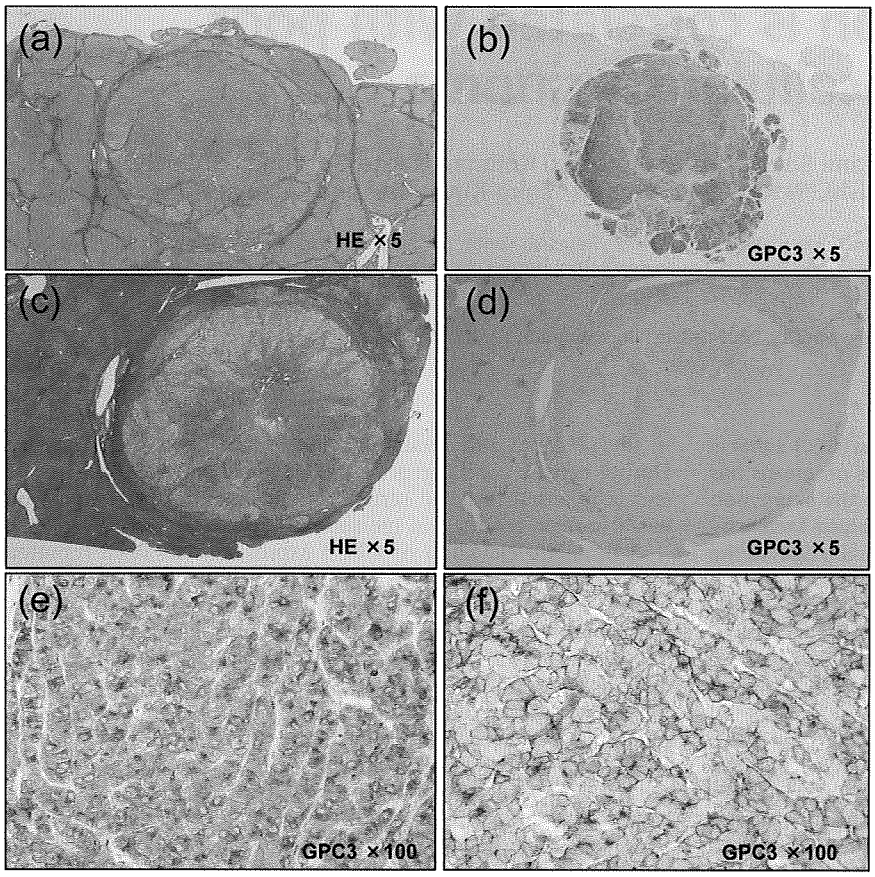

Fig. 1. Glypican (GPC)-3 expression and localization is hepatocellular carcinoma (HCC)-specific. (a,c) Microscopic view of a HE-stained sections of resected HCC. (b,d) HCC sections were stained for GPC3 expression with anti-GPC3 monoclonal antibody. (e) HCC displays prominent bile-canalicular immunostaining. (f) Membranous and cytoplasmic staining of liver tumor cells are shown.

peroxidase (DakoCytomation, Carpenteria, CA, USA), was used as the secondary antibody for 30 min at room temperature followed by three washes in TBS–Tween 20 for 5 min each. Finally, the visualization signal was developed by the addition of 3,3-diaminobenzidine tetrahydrochloride (DakoCytomation) to each slide, followed by incubation for 2 min. Slides were then washed in distilled water, counterstained with hematoxylin, and dehydrated.

For the immunohistochemical analysis of GPC3, we evaluated only the area of GPC3-positive staining in one slide in each patient, including the HCC lesion and adjacent non-cancerous lesion. At first, to analyze GPC3 expression, the results of immunohistochemical staining were classified according to the area of GPC3-positive staining cells as follows: –, negative (<10%); +/–, weakly positive (10–30%); and +, positive (>30%). Finally, in this study, we classified two groups between GPC3-negative (<10%) and GPC3-positive (>10%). The expression of GPC3 was judged to be positive when the percentage of immunoreactive cells was semiquantitatively assessed as being ≥10% in focal lesions. The slides were examined independently by two observers (H. Shirakawa and T. Nakatsura) and then collectively by a pathologist (M. Kojima).

Analysis of the correlation of GPC3 expression with various clinicopathological factors. The correlation of GPC3 expression with various clinicopathological factors was analyzed. Overall survival was calculated from the date of surgery to the date of death.

Statistical analysis. The differences in the level of GPC3 expression were tested by the χ^2-test and the means of each subgroup were compared using Student's *t*-test. Survival analyses were carried out according to the Kaplan–Meier method and the differences were assessed using the log-rank test. Follow-up time was censored if the patient was lost to follow up. Cox proportional-hazards analysis was used for univariate and multivariate analyses to explore the effects of the variables on survival. *P*-values of less than 0.05 were considered to be significant.

Results

Glypican-3 expression in HCC. In order to characterize the expression of GPC3 in HCC, 107 surgical specimens were analyzed immunohistochemically. The mean and median follow-up period were 3.4 ± 2.0 years and 3.5 years respectively. GPC3 expression was detected in 87 of the surgically resected tumor specimens (81.3%) (Fig. 1a,b), but not in the remaining 20 specimens (18.7%) (Fig. 1c,d). In most of the GPC3-positive cases, the protein expression was localized mainly in the cellular cytoplasm (Fig. 1e) with some amount detected on the cell membrane (Fig. 1f). The results of the immunohistochemical analysis were evaluated in relation to the pathological findings and follow-up data. There was no correlation between GPC3 expression and any of the clinicopathological features, except that the GPC3 expression increased with increasing degree of dedifferentiation of the cancer cells (Table 1). GPC3 expression was less frequently observed in well-differentiated HCC than in moderately or poorly differentiated HCC; the difference in frequency was statistically significant. Thus, an increase in GPC3 expression was correlated with increasing aggressiveness of the cancer cells, which was accompanied by dedifferentiation of the cells.

Correlation between GPC3 expression and patient survival. In order to determine the prognostic value of GPC3, the overall survival was compared between GPC3-positive and GPC3-negative HCC patients. The GPC3-positive HCC patients had a significantly lower 5-year survival rate than the GPC3-negative HCC patients (54.5 vs 87.7%, $P = 0.031$; Fig. 2a). After surgery,

doi: 10.1111/j.1349-7006.2009.01206.x

Table 1. Correlation between glypican (GPC)-3 expression and clinicopathological features of patients with hepatocellular carcinoma

Variable	GPC3 expression		*P*-value
	Positive (n = 87)	Negative (n = 20)	
Age (years) (mean ± SD)	63.6 ± 9.7	60.2 ± 11.8	0.169
Sex (male/female)	67/20	18/2	0.321
HBsAg status (positive/negative)	26/61	3/17	0.283
HCV status (positive/negative)	50/37	12/8	0.999
ICG R15 (%) (mean ± SD)	15.9 ± 8.1	15.5 ± 7.6	0.823
AFP (ng/mL) (mean)	6710	463	0.198
PIVKA-II (mAU/mL) (mean)	7370	5900	0.823
Tumor occurring (primary/recurrence)	64/23	16/4	0.753
Number of tumor (solitary/multiple)	64/23	11/9	0.172
Resection procedure (trisegmentectomy, lobectomy, or segmentectomy/subsegmentectomy or partial resection)	22/65	7/13	0.378
Operation time (min.) (mean ± SD)	310 ± 165	263 ± 119	0.248
Intraperative blood loss (mL) (mean)	2910	1500	0.356
Perioperative transfusion (present/absent)	45/42	9/11	0.767
Tumor size (mm) (mean ± SD)	54.7 ± 41.9	53.0 ± 31.2	0.861
Histological tumor differentiation (well/moderately and poorly)	6/81	6/14	0.032
pStage (UICC) (I/II/III)	35/41/11	6/10/4	0.577
Portal vein involvement (present/absent)	39/48	8/12	0.885
Hepatic vein involvement (present/absent)	9/78	1/19	0.750
Bile duct involvement (present/absent)	11/76	1/19	0.557
Intrahepatic metastasis (present/absent)	18/69	6/14	0.545
Non cancerous tissue (cirrhosis/non-cirrhosis)	36/51	4/16	0.075
Postoperative recurrence (present/absent)	70/17	16/4	0.963

AFP, alpha-fetoprotein; HBsAg, hepatitis B s antigen; HCV, hepatitis C virus; ICG-R15, indocyanine green-retention at 15 min; PIVKA-II, protein induced by vitamin K absence II; UICC, International Union against Cancer.

HCC recurrence was observed in 86 (80.4%) of the 107 patients. In the majority (97.7%) of patients with recurrence, the recurrence was observed in the residual liver. Among these 86 patients, 43 (50%) and seven (8.1%) developed multinodular and extrahepatic recurrence respectively. Although no correlations were observed between these recurrence patterns and GPC3 expression, GPC3 can only be used as an indicator of poor overall survival in HCC patients.

Among 80 of the 107 (74.6%) patients with initial treatment who underwent hepatectomy, none of the GPC3-negative HCC patients (n = 16, 20.0%) died during the follow-up period (Fig. 2b). The mean and median follow-up periods were 3.7 ± 2.1 and 3.7 years respectively. The 1-, 3-, and 5-year survival rates of the GPC3-positive HCC group were 84.4, 62.5, and 32.8% respectively. With regard to the tumor grade of HCC, 9 (11.3%) of the 80 patients with well-differentiated tumors showed significantly better prognosis without any record of deaths, compared with 71 (88.7%) patients with moderately and poorly differentiated HCC (Fig. 2c).

Further, among the 71 initial treatment patients who underwent hepatectomy and were found on histopathological examination to have moderately and poorly differentiated HCC, there were no deaths during the follow-up period in the GPC3-negative HCC group (Fig. 2d). The mean and median follow-up periods were 3.6 ± 2.0 and 3.6 years respectively.

Univariate and multivariate analyses to identify the prognostic variables in HCC patients. To identify the variables of potential prognostic significance in all the patients with HCC, univariate analysis of each variable was carried out in relation to the survival time. The difference in the prognosis was assessed by examining the relative hazard and *P*-value for each variable. The relative importance of each variable was then determined by multivariate Cox proportional hazards model analysis. Univariate analysis with stepwise inclusion of variables in the model revealed that the significant prognostic factors were GPC3 expression status, hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, indocyanine green-retention at 15 min (ICG-R15), serum protein induced by vitamin K absence II (PIVKA-II), tumor occurence, number of tumors, resection volume, pathological bile duct involvement, and pathological intrahepatic metastasis (Table 2). However, the multivariate analysis identified only GPC3 expression (P = 0.034), intrahepatic metastasis (P = 0.027), and multiple tumors (P = 0.006) as the independent prognostic factors related to overall survival (Table 2).

Discussion

In this study, we characterized the association between the expression level of GPC3 and the malignancy grade, and the prognostic value of GPC3 in HCC. Higher levels of GPC3 expression were observed in moderately or poorly differentiated tumor cells, which was in agreement with previous reports.[19] Our contingency table analysis showed that the GPC3 expression level was correlated with the tumor differentiation level. In addition, Kaplan–Meier survival analysis revealed that GPC3 expression was significantly linked to a poor prognosis after surgical resection in HCC patients. Moreover, univariate analysis indicated that GPC3 expression is associated with an increased risk of death from HCC, and this risk factor could still be extracted in a multivariate setting. On the other hand, multivariate analysis did not identify the tumor differentiation level as an independent predictive factor of the prognosis. Among the 80 HCC patients who underwent initial surgical treatment, the GPC3-negative patients showed better prognosis than the GPC3-positive patients. Patients with well-differentiated HCC also showed a better prognosis than those with moderately and poorly differentiated HCC. Furthermore, we confirmed that among the previously treated subjects, the GPC3-negative group had a better prognosis than the GPC3-positive group with moderately and poorly differentiated HCC tumors.

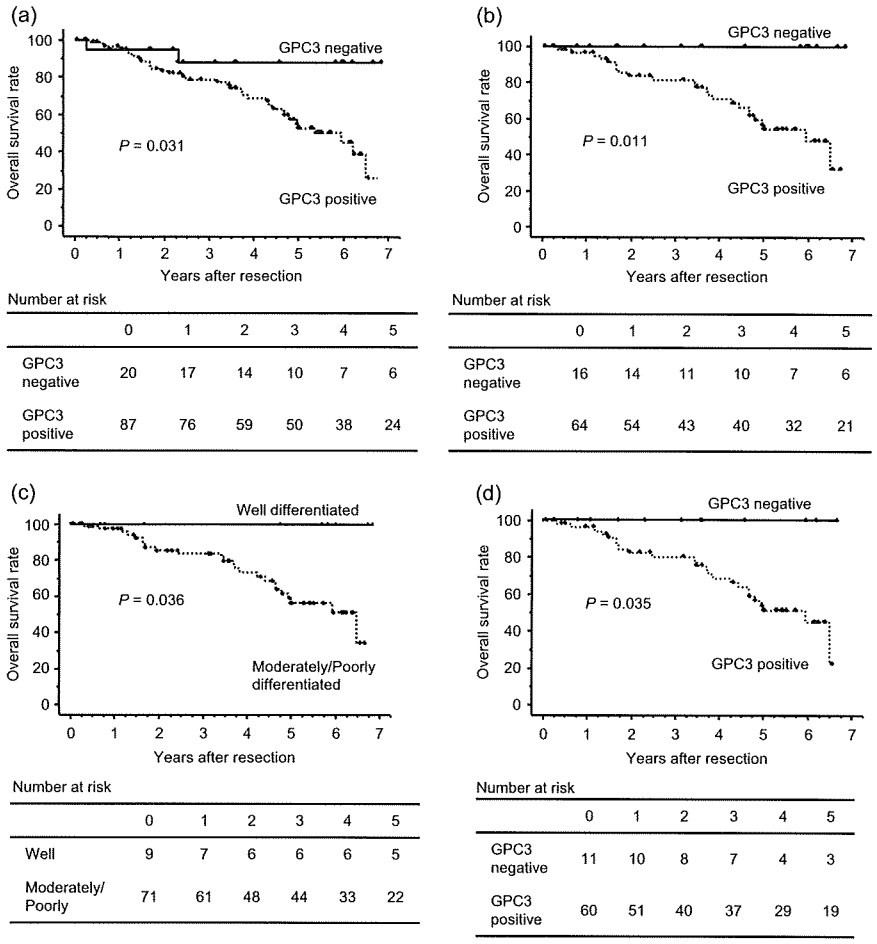

Fig. 2. Overall survival curves for the 107 hepatocellular carcinoma (HCC) patients stratified into those with glypican (GPC)-3-positive and GPC3-negative HCC. (a) Overall survival of patients with GPC3-positive HCC was shorter than those with GPC3-negative HCC ($P = 0.031$). (b) Overall survival curves in 80 of 107 HCC patients with initial treatment who underwent hepatectomy with positive and negative GPC3 expression. Patients with GPC3-positive HCC had a lower 5-year survival than those with GPC3-negative HCC ($P = 0.011$). (c) Overall survival curves in the 71 HCC patients with initial hepatectomy who exhibited well- and moderately and poorly differentiated HCC on histopathological examination. The 5-year survival rate was lower in the moderately and poorly differentiated GPC3-positive HCC than in the corresponding GPC3-negative HCC ($P = 0.036$). (d) Overall survival curves in the 71 initial treatment patients who underwent hepatectomy and exhibited moderately and poorly differentiated HCC on pathological examination with positive and negative GPC3 expression. The 5-year survival rate was lower in the GPC3-positive HCC patients than in the GPC3-negative HCC patients ($P = 0.035$).

Table 2. Prognostic factors for overall survival by univariate and multivariate analyses

Variable	No. patients	Univariate analysis		Multivariate analysis		
		5-year survival rate (%)	*P*-value	RR	95% CI	*P*-value
Age (years) (≥65/<65)	51/56	65.8/53.4	0.531			
Sex (male vs female)	85/22	56.1/72.7	0.403			
HBsAg (positive vs negative)	29/78	51.0/62.3	0.011	1.14	0.31–4.16	0.844
HCV (positive vs negative)	62/45	66.7/46.4	0.004	2.41	0.75–7.69	0.138
ICG R15 (%) (≥15 vs <15)	50/57	70.3/46.8	0.047	0.69	0.31–1.54	0.362
AFP (ng/mL) (≥50 vs <50)	45/62	49.1/65.1	0.132			
PIVKA-II (mAU/mL) (≥700 vs <700)	30/77	35.0/65.6	0.016	1.91	0.730–5.02	0.188
Tumor occurring (first vs recurrence)	80/27	62.8/50.2	0.019	1.83	0.78–4.31	0.167
No. tumors (solitary vs multiple)	75/32	65.7/42.7	0.009	3.53	1.41–8.00	0.006
Resection (trisegmentectomy, lobectomy, or segmentectomy/subsegmentectomy or partial resection)	29/78	36.5/67.1	0.005	1.71	0.52–5.60	0.374
Operation time (min) (>300 vs ≤300)	49/58	43.9/72.3	0.053			
Intraoperative blood loss (mL) (≥1300 vs <1300)	42/65	42.3/68.8	0.097			
Perioperative transfusion (present vs absent)	54/53	49.6/66.5	0.599			
Tumor size (mm) (>50 vs ≤50)	38/69	51.5/62.5	0.154			
Histological differentiation (well vs moderately and poorly)	12/95	77.8/56.4	0.102			
pStage (I vs II/III)	41/66	64.2/56.5	0.071			
Portal vein involvement (present vs absent)	47/60	64.9/58.5	0.369			
Hepatic vein involvement (present vs absent)	10/97	44.4/60.5	0.060			
Bile duct involvement (present vs absent)	12/95	20.0/62.7	0.004	0.94	0.31–2.85	0.912
Intrahepatic metastasis (present vs absent)	24/83	29.0/66.6	0.001	3.57	1.13–10.50	0.027
Non-cancerous lesion (cirrhosis vs non-cirrhosis)	40/67	53.6/61.9	0.232			
GPC3 staining (positive vs negative)	87/20	54.5/87.7	0.025	5.26	1.13–24.39	0.034

AFP, alpha-fetoprotein; CI, confidence interval; HBsAg, hepatitis B s antigen; HCV, hepatitis C virus; ICG-R15, indocyanine green-retention at 15 min; PIVKA-II, protein induced by vitamin K absence II; RR, relative risk; UICC, International Union against Cancer.

doi: 10.1111/j.1349-7006.2009.01206.x

In this study, the patients who were HCV positive, had higher ICG-R15 values, or portal vein involvement showed longer survival times, especially the patients who were HCV-positive or had higher ICG-R15 values, showed statistical significance in the univariate analysis. However, there was no statistical significance in these variables in the multivariate analysis. The reasons for these contradictive results in the univariate analysis are unclear.

In contrast, subgroup analysis did not reveal any significant difference in the disease-free survival rate between the GPC3-positive and GPC3-negative HCC patients (data not shown). The rate of recurrence in patients after surgery was 63.8% within the first 2 years after surgery among the previously treated patients in this study. Tumor recurrence in the GPC3-positive HCC patients occurred earlier than that in the GPC3-negative HCC patients until 9.7 months after the surgery among the patients who had received previous treatment. Two mechanisms of postoperative recurrence of HCC have been suggested: one is intrahepatic metastasis in the residual liver in a metachronous manner, and the other is multicentric hepatocarcinogenesis based on chronic hepatitis.[20–23] Some authors have suggested that early recurrence arises most often from intrahepatic metastases, whereas late recurrence is more likely to be multicentric in origin. Poon *et al.* and Portolani *et al.* reported that tumor factors like neoplastic vascular infiltration, but not host factors, were linked to early recurrence, whereas the risk of late recurrence was dependent on the underlying liver status.[21,22] These results indicate that GPC3 expression may indicate a high risk of intrahepatic recurrence.

Most of the GPC3 expression patterns in HCC cells showed the cytoplasmic pattern. There was no case that showed only the membrane pattern. Almost half of the HCC cases showed the mixed pattern (cytoplasm and membrane) and the other half showed only the cytoplasmic pattern.

There was no statistical significance between the mixed pattern (cytoplasm and membrane) and cytoplasmic pattern ($P = 0.297$) in Kaplan–Meier survival analysis. The functional difference between cytoplasmic GPC3 and membrane GPC3 is unknown, so further investigations are needed to clarify whether the different localization of staining has a different significance.

In addition to the investigation of its role as a prognostic indicator, a phase I clinical trial of a GPC3-derived peptide vaccine for advanced HCC is now underway; GPC3 is an ideal target for this therapy because it is more effective in patients with increased expression of GPC3, which is frequently observed in the later stages of HCC, as shown in the present study. The poor prognosis of patients with GPC3-positive HCC also prompted us to develop a strategy of anticancer immunotherapy,[24,25] that is, we may expect the effect of hepatocarcinogenesis prevention after surgery in patients with GPC3-positive HCC.

In summary, our study evaluated the prognostic significance of GPC3 expression at the protein level in clinical tissue specimens of HCC. The overall survival rate was significantly poorer in patients with elevated GPC3 expression in the tumor than in those with lower levels of GPC3 expression. Further functional characterization of GPC3 may be expected to lead to a better understanding of the molecular mechanisms underlying the development and progression of HCC.

Acknowledgments

This work was supported in part by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labor, and Welfare, Japan, a Grant-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour, and Welfare, Japan, and awardee of research Resident Fellowship from the Foundation for Promotion of Cancer Research (Japan) for the Third-Term Comprehensive 10-Year Strategy for Cancer Control (H.S.).

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