分類される（表13）.

切除後の予後因子については，切除断端のがん陽性，リンパ節転移，血管浸潤，腫瘍数などが報告されている.

F. 治療法

外科切除が唯一の根治的治療法であり，標準治療である．しかし，肝細胞がんの肝硬変のような高危険群がみられず，早期症状に乏しいため早期発見が難しい．したがって，腹痛や黄疸など症状が発現してから診断されることが多く，切除不能の場合が多い．第17回全国原発性肝癌追跡調査報告によると，主な治療法は切除68.3%，薬物療法19.6%，塞栓療法4.5%，局所療法2.3%と報告されている.

切除不能例の治療法としては薬物療法や局所療法などが行われるが，信頼の高い成績は報告されていない．これらの非切除治療に十分なエビデンスはない.

G. 予　後

第17回全国原発性肝癌追跡調査報告では，全症例の生存率は，1年49.2%，5年19.6%，肝切除例では1年70.5%，5年32.7%，非切除例では1年59.5%，5年17.4%と報告されている.

◈参考文献

1) 日本肝癌研究会(編)：臨床・病理 原発性肝癌取扱い規約，第5版，金原出版，東京，2008
2) 日本肝癌研究会追跡調査委員会：第17回全国原発性肝癌追跡調査報告 2002-2003．肝臓 **48**：117-140, 2007
3) 科学的根拠に基づく肝癌診療ガイドライン作成に関する研究班：科学的根拠に基づく肝癌診療ガイドライン，2005年版，金原出版，東京，2005
4) Bruix J, Sherman M, Llovet JM et al : Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. J Hepatol **35** : 421-430, 2001
5) TNM Classification of malignant tumours : Liver, 6th Ed, Sobin LH, Wittekind Ch(eds), UICC, Wiley-Liss, New York, pp.82-83, 2002
6) Llovet JM, Ricci S, Mazzaferro V et al : Sorafenib in advanced hepatocellular carcinoma. N Engl J Med **359** : 378-90, 2008
7) Kanematsu T, Furui J, Yanaga K et al : A 16-year experience in performing hepatic resection in 303 patients with hepatocellular carcinoma : 1985-2000. Surgery **131** : S153-158, 2002
8) Livraghi T, Solbiati L, Meloni MF et al : Treatment of focal liver tumors with percutaneous radio-frequency ablation : complications encountered in a multicenter study. Radiology **226** : 441-451, 2003
9) Camma C, Schepis F, Orlando A et al : Transarterial chemoembolization for unresectable hepatocellular carcinoma: meta-analysis of randomized controlled trials. Radiology **224** : 47-54, 2002
10) Kudo M, Chung H, Osaki Y : Prognostic staging system for hepatocellular carcinoma (CLIP score) : its value and limitations, and a proposal for a new staging system, the Japan Integrated Staging Score (JIS score). J Gastroenterol **38** : 207-215, 2003

3 標準的化学療法

Don't Forget!

- □ 食道がんでは術前補助化学療法(5FU+ シスプラチン)が効果を上げている.
- □ 胃がんでは S-1 ベースの薬物療法が主体となっている.
- □ 切除不能大腸がんでは分子標的薬(ベバシズマブ, セツキシマブ)併用が有用である.
- □ 膵がんではゲムシタビンに加えて S-1 が期待されている.
- □ 局所治療が困難な肝細胞がんではソラフェニブが保険承認となった.

化学療法の適応は, 全身状態が良好であることが前提で, パフォーマンスステータス(PS)が良好かつ臓器機能が保たれていて十分なインフォームド・コンセントが得られていることが重要である. 標準的化学療法(標準治療)とは,「第 III 相試験などのエビデンスレベルが高いデータが揃っていて, 生存期間・副作用などでベネフィットが期待されると結論づけられ, 現時点で最も勧められるべき治療法」をいう. 通常, 第 III 相試験で得られた結果は, 厳格な選択基準を満たした症例を対象として得られたものであるため, 通常の実地臨床においては, 1 症例ごとに標準治療が存在するのかを常に意識することが大切である. 例えば, 治癒切除例に対する術後補助療法と切除不能・再発症例に対する薬物療法の意義を混同しないように注意する. また, 実際には全身状態が良好である対象とは限らず, PS・臓器機能・合併症などの患者情報から適応を十分に考慮する. 必要ならば投与量・スケジュールの調節を行って治療の個別化を検討するが, 安易な個別化は治療効果を損ねる可能性もあり, 十分な説明・同意が必要である. 抗腫瘍薬は治療域の狭い薬剤であり, 効果と毒性の薬理学的特性を十分に把握しながら治療にあたる. 最近では外来で薬物療法を行う施設も増えており, 専門薬剤師・専門看護師とも情報共有を行い, 適切な副作用モニタリングと対応が必須である.

1 食道がん

食道がんでは, 病期分類による治療アルゴリズムが日本食道学会から提唱されている. stageII ～ III では手術単独療法が標準治療と考えられていたが, 最近の臨床試験のデータにより, 治療オプションの選択肢が増えている. 手術可能な stageII ～ III 症例に対して, 日本臨床腫瘍研究グループ(JCOG)が行った第 III 相試験(JCOG 9204)では手術単独と術後補助化学療法を比較し, 5 年無再発生存率で術後補助化学療法の優越性が示された. さらに, この術後補助化学療法と術前補助化学療法を比較した第 III 相試験(JCOG 9907)では, 無増悪生存期間, 5 年生存率において術前群で有意に良好との結果が得られ, わが国では術前補助化学療法が新しい標準治療として有望である. stageI ～ III において, 手術拒否および臓器機能低下などで手術に耐えられない場合には, 根治的放射線化学療法(5FU + シスプラチン + 50.4 ～ 60 Gy)が考慮される. stageII ～ III 症例(T4 は除く)を対象に 5FU + シスプラチンに放射線 60 Gy を同時併用した第 II 相試験(JCOG

9906)では完全奏効割合62%，5年生存率は37%であり，心嚢水，胸水などの晩期毒性の問題はあるものの根治的放射線化学療法はオプションの1つと考えてよい．StageIIIのうち局所浸潤を伴うT4症例およびstageIVaでは放射線化学療法を考慮する．stageIVb(遠隔転移)では化学療法を行うが，食道狭窄を伴う場合には緩和的放射線療法の併用を考慮する．stageIVb症例を対象とした第II相試験の結果では生存期間中央値は7か月程度であり，新薬などを用いた新規治療の開発が望まれている．2次治療としてはドセタキセルが考慮されるが，わが国で行われた第II相試験での奏功率は17%と低く，標準治療は確立していない．その他，ネダプラチンやフィルデシン®が使用可能ではあるが，十分なインフォームド・コンセントを得たうえで使用を考慮する．

2 胃がん

わが国の胃がんの診断学・外科治療学は世界的にみても洗練されており，さらに化学療法においても第III相比較試験が最近相次いで報告され，独自の進展をみせている．

a 術後補助化学療法

治癒切除可能胃がんの標準治療は手術であるが，stageII～III症例では手術単独では不十分と考えられ，術後の補助化学療法の有用性を示すエビデンスが蓄積されてきている．米国のINT 0116試験，欧州のMAGIC試験ではそれぞれ化学放射線療法(5FU＋ホリナートカルシウム＋放射線45 Gy)，ECF(エピルビシン＋シスプラチン＋5FU)併用療法の有用性が示唆された．わが国からはACTS-GC試験[1]として，D2郭清による治癒切除stageII～III症例を対象に，手術単独群と術後にS-1(テガフール＋オキソン酸配合剤)を1年間内服する群に割り付ける比較試験が行われており，S-1群の有用性が示されている．ACTS-GC試験は手術の品質管理や選択基準などにおいてクオリティーが高いと考えられ，さらに胃がん治療の国ごとの多様性を考慮すると，わが国においては必ずしも欧米の結果を外挿する必要はないと思われる．わが国ではACTS-GC試験の結果をもって，stageII～III胃がんに対してS-1による術後補助化学療法が標準的な治療と考えてよい．今後はS-1ベースの併用レジメンや新規分子標的薬を用いた術後補助療法の臨床試験が企画されており，さらには術前の補助化学療法により腫瘍縮小を図る臨床試験も展開されており，結果が期待される．

b 切除不能・再発胃がん

2007年に日本から重要な第III相比較試験の結果が報告された．JCOG 9912試験[2]は5FU持続静注法を対照とし，S-1の非劣勢とイリノテカン＋シスプラチン併用療法の優越性を検証する試験で，S-1の非劣勢が証明された．また，SPIRITS試験[3]ではS-1単独療法とS-1/シスプラチン併用療法の比較が行われ，S-1/シスプラチン併用療法の優越性が証明された．これらの結果を合わせると，わが国では切除不能胃がんの標準的化学療法はS-1/シスプラチン併用療法と考えることができる．世界的には5FUやカペシタビンにプラチナ製剤を加えるレジメンが広く用いられており，PS不良例などでは5FUベースの治療を考慮する．現在，カペシタビン＋シスプラチンに分子標的薬であるトラスツズマブやベバシズマブを上乗せする国際共同第III相比較試験が進行中であり，その結果によって，1次治療に分子標的薬が加わる可能性がある．一方，2次治療以降のエビデンスは乏しいが，初回治療例を対象としたV325試験で，5FU＋シスプラチン併用療法にドセタキセルを加えた3剤併用療法の優越性が示されており，実地臨床ではタキサン系薬剤(ドセタキセル/パクリタキセル)を2次治療で使用することが行われている．このほか，イリノテカンも使用可能であるが，消化管

閉塞・黄疸症例では禁忌であるため注意が必要である.

3 肝細胞がん

肝細胞がんに対する化学療法としては,局所療法としての肝動脈化学塞栓療法・経動脈化学療法や全身化学療法が考慮される.肝がん診療ガイドラインによれば,化学療法は推奨グレード分類では行うことを考慮してもよいが十分な科学的根拠がないC1に分類されていて,局所療法以外の標準的化学療法は確立していなかった.一方,2007年にチロシンキナーゼ阻害薬であるソラフェニブとプラセボによる第III相比較試験(SHARP試験)の結果が報告され[4],生存期間中央値でソラフェニブ群で10.7か月,プラセボ群で7.9か月で,有意にソラフェニブ群で良好であった.その後にAsia-Pacific地域での第III相試験も行われ,同様にソラフェニブの有効性が示された.わが国でも2009年6月に承認され,局所治療が困難な肝細胞がんでは標準的治療となる可能性が高い.

4 膵がん

a　術後補助化学療法

2007年に報告されたCONCO-001試験によれば,膵がん術後のゲムシタビンによる術後補助化学療法群で無再発生存期間,全生存期間の延長がみられた.わが国でも厚生労働省研究班によるゲムシタビンによる術後補助化学療法と術後経過観察群のランダム化比較試験が行われ,ゲムシタビンの有用性が報告されている.現時点では膵がん治癒切除後の術後療法として実地臨床ではゲムシタビンが用いられている.

b　切除不能・再発膵がん

ゲムシタビンが症状緩和効果,延命効果を示すことが報告されて,標準的治療と位置づけられている.さらに,ゲムシタビンに様々な薬剤を上乗せした併用治療の有用性を検討する比較試験も行われており,エルロチニブ併用療法が良好な結果を示したが,コントロールと比較して生存期間中央値の差はわずかであったため,いまだにゲムシタビンを超える標準治療は確立していないと考えられている.2次治療としては,わが国ではS-1が使用可能である.初回治療例を対象にゲムシタビン単独とS-1単独療法,ゲムシタビン+S-1併用療法の3群比較の第III相試験が進行中であり,結果が期待されている.

5 胆道がん

欧米では発生頻度が低く,症例数に限界があるために,現在までに標準的化学療法が確立していない.胆道がんと組織学的に類似していると考えられている膵がんで有効性が認められているゲムシタビンおよびS-1がわが国では保険承認されており(奏効率はそれぞれ17.5%,21%),実地臨床として使用可能である.ゲムシタビンを対照としてゲムシタビン+シスプラチン併用療法を試験治療とした第III相比較試験(ABC-02)が英国で行われ,併用治療で生存期間の延長が報告された[5].今後は,切除不能胆道がんの標準的治療に位置づけられていくと思われる.

6 大腸がん

a　術後補助化学療法

治癒切除大腸がんでは,術後補助療法の再発抑制効果と生存期間延長が示されている.5FU + leucovorin(RPMIレジメン)を6か月行うこととされているが,経口薬tegafur・uracil/leucovorin,カペシタビンの非劣性も示されており,いずれかを半年間行う.欧米ではFOLFOX4(leucovorin+5FU+oxaliplatin)の有用性が報告されていて,わが国でも2009年秋に保険適用となったが,欧米とわが国の治療成績の違いや神経毒性を考慮すると,そのまま外挿する

ことには議論の余地がある.

b　切除不能・再発大腸がん ……

切除不能大腸がんに対する初回治療としてIFL(5FU + leucovorin + irinotecan 急速静注法)に抗VEGF抗体のベバシズマブ(beva)を上乗せするレジメンとIFL +プラセボ, 5FU + leucovorin + bevaの3群を比較した第III相試験(AVF2107g試験)[6]が行われ, 全生存期間においてIFL + bevaの優越性が示された. また, 2次治療としてFOLFOX4単独群とFOLFOX4 + beva群で行われた比較試験(ECOG 3200)でもFOLFOX4 + beva群において全生存期間の延長がみられたことにより, bevaの初回・2次治療の有用性が示唆された. これらを踏まえて, 現在では, 初回治療としてはmFOLFOX6あるいはFOLFIRI(leucovorin + 5FU + irinotecan)のいずれかにbevaを併用する方法が標準と考えられる. FOLFOX/FOLFIRI法は2日間の持続点滴が必要であり, 中心静脈ルートへのポート留置を行い, 外来で投与可能である. bevaに特徴的な副作用として高血圧・出血・血栓症・蛋白尿・消化管穿孔などがある. GRECOR試験(FOLFIRI → FOLFOX対FOLFOX → FOLFIRIの第III相比較試験)では, 生存期間中央値は20か月を超えており, これらの薬剤を順次使い切ることが重要と考えられている. 1次治療でFOLFIRI + bevaを選択した場合には2次治療ではFOLFOX, 1次治療でFOLFOX + bevaを選択した場合には2次治療ではFOLFIRIあるいはイリノテカン単剤を用いる. なお, bevaは3次治療以降での有用性は乏しいとされ, 前期治療(1次もしくは2次治療)で用いることが推奨される. 抗EGFR抗体のセツキシマブは2次治療以降において, 単独もしくはイリノテカン併用療法として考慮する. セツキシマブは*KRAS*遺伝子の変異型では効果が乏しいとされ, 欧州では*KRAS*遺伝子野生型のみが適応となっているが, わが国では, 一部の施設でのみ先進医療として遺伝子検査を行っている. また, イリノテカンに関しては*UGT1A1*遺伝子の多型(*UGT1A1*28*および*UGT1A1*6*)を有する場合には好中球減少のリスクを高めるとの報告があり, わが国でも検査キットが保険承認されている. これら遺伝子検査の重要性は認識されつつあるが, 遺伝的背景を踏まえたわが国での情報は十分とはいえず, 実地診療における個別化医療は今後の課題といえる.

7 消化管間質腫瘍(gastrointestinal stromal tumor : GIST)

GISTは腫瘍細胞にある細胞膜のKITやPDGFRα蛋白の異常を伴うことが分かっている. イマチニブは慢性骨髄性白血病の原因であるBCR-ABL蛋白を特異的に阻害する目的で開発された分子標的薬であるが, KIT, PDGFRを標的として結合し, チロシンキナーゼ活性を阻害する. GISTに対して初回治療薬として用いられる. 最近ではイマチニブ耐性例に対する2次治療薬としてスニチニブが承認されている.

文献

1) Sakuramoto S, *et al.* : *N Engl J Med* 2007 ; **357** : 1810-1820
2) Boku N, *et al.* : *J Clin Oncol* 2008 supple
3) Koizumi W, *et al.* : *Lancet Oncol* 2008 ; **9** : 215-221
4) Llovet JM, *et al.* : *N Engl J Med* 2008 ; **359** : 378-390
5) Valle JW, *et al.* : *J Clin Oncol* 2009 **27** supple : 202s, (abstr 4503)
6) Hurwitz H, *et al.* : *N Engl J Med* 2004 ; **350** : 2335-2342

杏林大学医学部内科学腫瘍内科　**長島文夫, 古瀬純司**

4 化学療法の副作用対策

Don't Forget!

- □ 副作用の多くは，早期モニタリングと適切な処置で対応可能である．
- □ 新規分子標的薬の副作用は従来の抗腫瘍薬の副作用とは異なるので注意が必要である．
- □ 抗腫瘍薬の副作用とがんの病態に伴う症状の鑑別に配慮する．
- □ 外来薬物療法においては，専門薬剤師・看護師などと連携した患者指導が重要である．

がん薬物療法における副作用として，従来から使用されてきた5FUやシスプラチンなど細胞傷害性薬剤にみられる骨髄抑制や消化器毒性など，対処法が確立しているもののほかに，最近になって臨床導入が進んだベバシズマブにみられる高血圧・蛋白尿・出血・血栓症，セツキシマブにおける皮膚障害などメカニズムそのものが十分に解明されていないものにも十分配慮する．これらの新規抗腫瘍薬では，わが国で承認後，安全性を確認する目的で大規模な使用成績調査が行われており，安全性情報を速やかに臨床現場に反映していく必要がある．外来薬物療法を行うにあたって，専門薬剤師・専門看護師と連携して患者指導を行い，早期モニタリングと適切な対応が必須である．

1 骨髄抑制(好中球減少，貧血，血小板減少)

ほとんどの抗腫瘍薬においてみられるもので，対応を誤ると致死的なものであり，慎重な対応が必要である．

①**発熱性好中球減少症**：白血球(好中球)減少は化学療法施行後7～14日程度で最低値(nadir)を示すことが多く，発熱をきたす場合は発熱性好中球減少症と呼ぶ．感染症を疑う場合には血液培養を行ったうえで，エンピリックセラピーとして広域スペクトラムの抗菌薬を速やかに開始する．抗菌薬の補助療法としてG-CSFをルーチンには使用しないが，予後不良因子がある場合(好中球減少の長期化・重症化が予想される場合，65歳以上，原疾患のコントロール不良など)はG-CSFの投与を考慮する[1]．また，幹細胞の増殖により抗腫瘍薬の感受性が高まるため，化学療法開始前にG-CSFを予防的に投与することは避けるべきとされている．ただし，好中球減少の頻度が高いレジメン使用時や好中球減少のために用量を下げる必要があり予後に影響を与える場合にはG-CSFの予防的投与を考慮するが，わが国において実地臨床で用いる消化器がん薬物療法において予防的にG-CSF投与を考慮せざるをえないレジメンは基本的にはないと考えられる．

②**貧　血**：抗腫瘍薬治療に伴うものとがんに伴うものがある．ヘモグロビン値の悪化が予後に影響を与えていること，QOLを大きく損ねることが示唆されているため，貧血の管理は重要である．対応としてわが国で実施可能なものは赤血球輸血であり，ヘモグロビン値7 g/dLを目安に輸血を考慮する．海外ではエリスロポエチン製剤による対応が確立しているが，

最近になって安全性や腫瘍進行に関する警告も追加されたため，その使用には十分配慮すべきである．

③**血小板減少**：がんの骨髄浸潤，凝固系の異常に伴うDICなどにより潜在的に血小板が減少している病態が存在し，さらに抗腫瘍薬投与に伴い血小板の低下をきたす．抗腫瘍薬投与後2～3週後に最低値になることが多く，消化器がんで用いる薬剤としてはゲムシタビン，シスプラチン，(マイトマイシンC，ネダプラチン，ビンデシン)などにより生じやすい．実際の輸血に関してはアレルギー反応や感染症の問題もあり，基礎疾患・症状などリスクベネフィットを考慮して適応を判断する．

2 消化器毒性(嘔気・嘔吐，下痢，便秘)

①**嘔気・嘔吐**：嘔気・嘔吐は嘔吐中枢が刺激されて出現するものと考えられており，出現時期により急性，遅発性，予期性に分類できる．消化器がん領域では，食道がん，胃がんにおけるシスプラチン，大腸がんにおけるイリノテカン，オキサリプラチン使用時には高頻度に症状が出現する．実際には，腹膜転移・腸閉塞・脳転移などの疾患依存性，モルヒネなどの薬剤依存性による嘔気なども併存することがあるため，全身状態の把握は重要である．具体的な対応としては，5-HT3受容体拮抗薬，デキサメタゾン4～20 mgを予防的に投与する[2]．症状が強く出現した場合はメトクロプラミド，プロクロルペラジン，ハロペリドールなどを順次追加する．これらの薬剤は反復投与が可能であるが，錐体外路症状(イライラする，落ち着かないなどの訴えが多い)が出現することがあるため注意する．

②**下　痢**：化学療法時の下痢にはその出現時期により，化学療法投与当日に起こるコリン作動性の下痢と，投与後数日以降に腸管粘膜が損傷されて起きる遅発性の下痢とに分類できる．遅発性の下痢が出現している時期には好中球減少が重なることもしばしば経験するため，致命的な状態を回避するためにも早期のモニタリング・対応が重要である．食道がん，胃がん，大腸がんにおけるフルオロウラシル(S-1を含む)，大腸がんにおけるイリノテカンを使用し，外来で経過観察する場合は特に注意する．水様性下痢が出現した場合は緊急受診を勧め，ロペラミドなどの止痢薬を予防的に処方するなど，対応を工夫する．

③**便　秘**：抗腫瘍薬により，末梢神経障害と自律神経障害により，腸管の運動制限が生じ，便秘が引き起こされると考えられており，パクリタキセル，フィルデシン® のほかにも，制吐薬である5-HT3受容体拮抗薬，モルヒネなどで便秘をきたしやすい．多くの場合は水分を多めに摂取し，緩下薬を使用して腸管運動を調節することで対応可能である．

3 末梢神経毒性

白金製剤(オキサリプラチン，シスプラチン)やタキサン系薬剤(パクリタキセル，ドセタキセル)では四肢末梢に起きる神経毒性がみられる．オキサリプラチンによる神経障害は，手足，咽頭部，口唇周囲のしびれや痛みとして出現し，オキサリプラチン投与中に出現する急性期のものと，投与を繰り返すことにより蓄積する慢性期毒性がある．進行すると機能障害(感覚運動協調が必要な運動が困難；ボタンが留められない，箸が持てない，字が書けない，痛くて歩けないなど)をきたし，休薬により軽減するが不可逆性のこともあるとされ，QOLを低下させる非常に厄介な毒性である．計画的に治療を休止するstop and go strategy[3]やオキサリプラチンの投与前後にカルシウム/マグネシウムを投与すること

で神経毒性を軽減する方法などが検討されている．タキサン系においても知覚障害が出現する．典型的にはタキサン投与直後に始まり次サイクル前には改善するが，症状の強さは，投与量・投与法に依存するといわれ，神経障害の合併症を有する糖尿病や腎臓病のある場合，シスプラチンの既治療例で増強するとの報告がある．いずれの薬剤においても症状の悪化がみられる場合は原因薬剤の減量，休薬を考慮する．

4 血管関連有害事象

血管関連有害事象として，抗腫瘍薬が血管外の周辺組織に漏れることにより周囲組織の炎症や壊死をきたす血管外漏出，抗腫瘍薬が血管を直接損傷させて炎症をきたす血管炎，漏出や血管炎に伴う血管痛などが出現する．特に，血管外漏出では潰瘍や壊死に至ることがあり，外来での経過観察には慎重な対応が必要である．抗腫瘍薬の種類により，皮膚障害の程度が異なるため，侵襲程度により起壊死性抗腫瘍薬(vesicant drug)，炎症性抗腫瘍薬，起炎症性抗腫瘍薬と3つに分類する．強い痛みと組織壊死をきたし，潰瘍形成に至ることもある起壊死性抗腫瘍薬として，消化器がん領域に関連する薬剤はタキサン系(ドセタキセル，パクリタキセル)，マイトマイシンC，ビンデシンなどがあげられる．これらの薬剤の漏出が疑われた場合は投与をただちに中止し，原因薬剤を可能な限り吸引し，ステロイドの局所皮下注射，ステロイド軟膏の塗布を行い，皮膚科の診察を依頼し外科的処置の対応も考慮する．局所の炎症を起こすが壊死には至らない炎症性抗腫瘍薬としてはフルオロウラシル，シスプラチン，オキサリプラチン，イリノテカン，ゲムシタビンなどがあり，これらの薬剤の場合は必要に応じてステロイド薬を投与する．

5 薬剤性肺障害

抗腫瘍薬使用時には，びまん性の間質性肺炎を呈することがあり，消化器がん薬物療法においてはゲムシタビン(1.4%)，イリノテカン(0.9%)，S-1(0.3%)，イマチニブ(5%以下)，ソラフェニブなどで十分に配慮する．薬剤投与歴と呼吸困難・咳嗽・発熱などの臨床症状から肺炎を疑い，胸部CTによる診断は必須である．抗腫瘍薬による間質性肺炎を疑った場合は，当該薬剤を速やかに中止し，ステロイドの投与も考慮する．また，既存の間質性肺炎を有する症例では抗腫瘍薬投与の適応は慎重に判断する．

6 アレルギー反応

オキサリプラチンでは10%以下の頻度ではあるがアレルギー反応が出現する．アナフィラキシーの報告も含まれており注意が必要である．この反応は初回投与時よりも数回施行後に起きることが多く，皮膚の発赤，掻痒感，皮疹などから呼吸困難，意識消失などの重篤な症状も出現するので注意が必要である．出現時は投与を中止し抗ヒスタミン薬，ステロイド薬を投与する．症状が軽度の場合は次回以降に，抗アレルギー薬の追加，投与時間の延長などで継続治療を検討するが，症状が強い場合は休薬，レジメン変更を検討する．

7 分子標的薬における副作用対策

分子標的薬では，その作用機序の特性から従来の細胞傷害性抗腫瘍薬とは異なった副作用が報告されている．

①ベバシズマブでは高血圧，蛋白尿，消化管穿孔(発現率は1～2%)，出血，動静脈血栓塞栓症など緊急対応を要する副作用に注意する．ベバシズマブ投与に伴う高血圧は本態性高血圧の発現メカニズムと異なっているとされ，蛋白尿の改善効

果も期待して，ACE 阻害薬やアンジオテンシン II 受容体拮抗薬が推奨されている．蛋白尿はベバシズマブ投与で多くみられ，多くは非重篤なものであるが定期的な尿チェックが必要である．高血圧・糖尿病などのリスク因子をもつ場合にはこれらのコントロールを行うことも重要である．ベバシズマブによる穿孔の主たる部位は下部消化管であるが，下部消化管以外にも発生している．消化管潰瘍，腫瘍壊死，憩室炎，腸閉塞，放射線照射，化学療法などに関連する腸炎，腹腔内の炎症が発現メカニズムとして考えられているので，これらを有する場合には注意する[4]．また，1 か月以内の大腸内視鏡検査歴がリスク因子になるとされ，ベバシズマブ治療中は内視鏡検査の時期にも配慮する．重篤な出血の頻度は 1.4% で，消化管出血による死亡例も含まれており，凝固系異常がある場合には注意深く観察する．脳転移例では禁忌とされ，空洞形成のある肺転移なども出血のリスクが高まるため，リスクとベネフィットを考慮する．一般に悪性腫瘍においては血液凝固系の異常が生じるとされ，深部静脈血栓症や肺塞栓症の発生頻度が高いことが知られている．ベバシズマブの副作用として，動脈血栓症(国内の特定使用成績調査で重篤なものは 0.3%)，静脈血栓症(同，1.3%)[5]があり下肢痛や胸腹部痛出現時は血栓症を鑑別する．

②セツキシマブでは皮膚病変，infusion reaction が特徴的である．セツキシマブに伴う皮膚障害の発生メカニズムは不明な点が多い．ざ瘡様皮膚炎はセツキシマブ投与 1 ～ 2 週間で出現することが多い．続いて皮膚乾燥や亀裂，爪囲炎をきたす．これら皮膚障害の程度と治療効果には相関があるとされるため，皮膚症状を適切にコントロールしながら治療を継続することがポイントである．ざ瘡様皮疹，爪囲炎に対してはステロイド外用薬，抗菌薬の軟膏，乾燥症に対しては保湿剤で対応する．セツキシマブはキメラ型抗体であり，15 ～ 20% の頻度で投与時の infusion reaction を起こす．infusion reaction とは点滴開始直後に起きる，有害事象の総称である．前投薬として抗ヒスタミン薬を投与する．発熱・悪寒・発疹などの軽度から中等度の症状が出現した場合には，投与速度を下げて継続を試みる．一方，血圧低下，意識レベル低下，気管支痙攣，じんま疹など重症例も 2 ～ 5% 存在し，アナフィラキシー反応に準じて緊急対応が必要である．このような場合には投与は中止とする．

③マルチキナーゼ阻害薬であるソラフェニブではリパーゼ・アミラーゼの上昇，発疹，手足症候群，下痢，高血圧などに注意する．特に手足症候群，下痢では重篤な例も認めており発生時は速やかに休薬することが重要である．皮膚症状に対しては皮膚刺激を軽減し，外用薬(ステロイド，保湿剤など)を使用する．

文献

1) Smith JT *et al.* : *J Clin Oncol* 2006 ; **24** : 3187-3205
2) がん診療レジデントマニュアル第 4 版
3) Tournigand C, *et al.* : *J Clin Oncol* 2006 ; **24** : 394-400
4) Management Guidelines for Bevacizumab-Related Side Effects in Patients with Colorectal Cancer. BC Cancer Agency
5) アバスチン市販直後調査　中外製薬ホームページ

杏林大学医学部内科学腫瘍内科　**長島文夫，古瀬純司**

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

HIROFUMI SHIRAKAWA[1,3], TOSHIMITSU KURONUMA[1], YOSHIKO NISHIMURA[1], TAKAHIRO HASEBE[2], MASAYUKI NAKANO[4], NAOTO GOTOHDA[3], SHINICHIRO TAKAHASHI[3], TOSHIO NAKAGOHRI[3], MASARU KONISHI[3], NOBUAKI KOBAYASHI[5], TAIRA KINOSHITA[3] and TETSUYA NAKATSURA[1]

[1]Section for Cancer Immunotherapy, Investigative Treatment Division, [2]Pathology Division, Research Center for Innovative Oncology, [3]Hepato-Biliary Pancreatic Surgery Division, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, 277-8577 Chiba; [4]Department of Pathology, Tokyo Women's Medical University Yachiyo Medical Center, 477-96 Owada-Shinden, Yachiyo, 276-8524 Chiba; [5]Department of Organ Regulatory Surgery, Ehime University Graduate School of Medicine, Shitsukawa, Toon, 791-0295 Ehime, Japan

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.

Correspondence to: Dr Tetsuya Nakatsura, Section for Cancer Immunotherapy, Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa 277-8577, Japan
E-mail: tnakatsu@east.ncc.go.jp

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

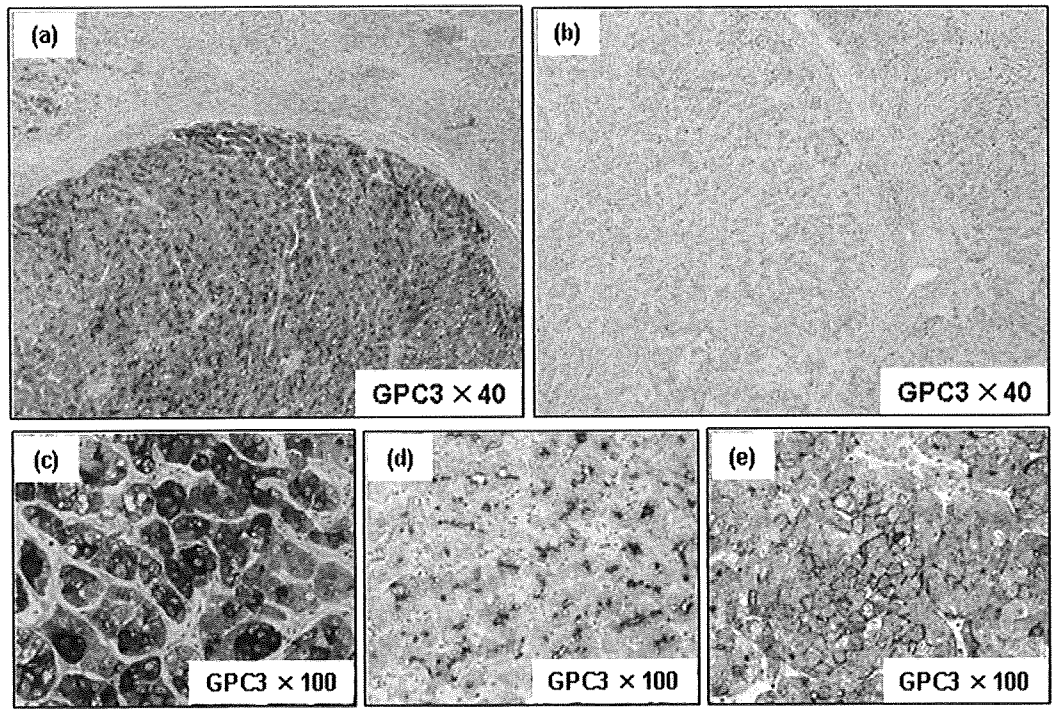

Figure 1. GPC3 expression was specific to HCC and absent in ICC. Immunohistochemical detection of GPC3 expression in HCC (a) and ICC (b) (magnification, x40). Immunostaining patterns of HCC: (c) diffuse in cytoplasm, gulanular in cytoplasm (d), and membranous (e).

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

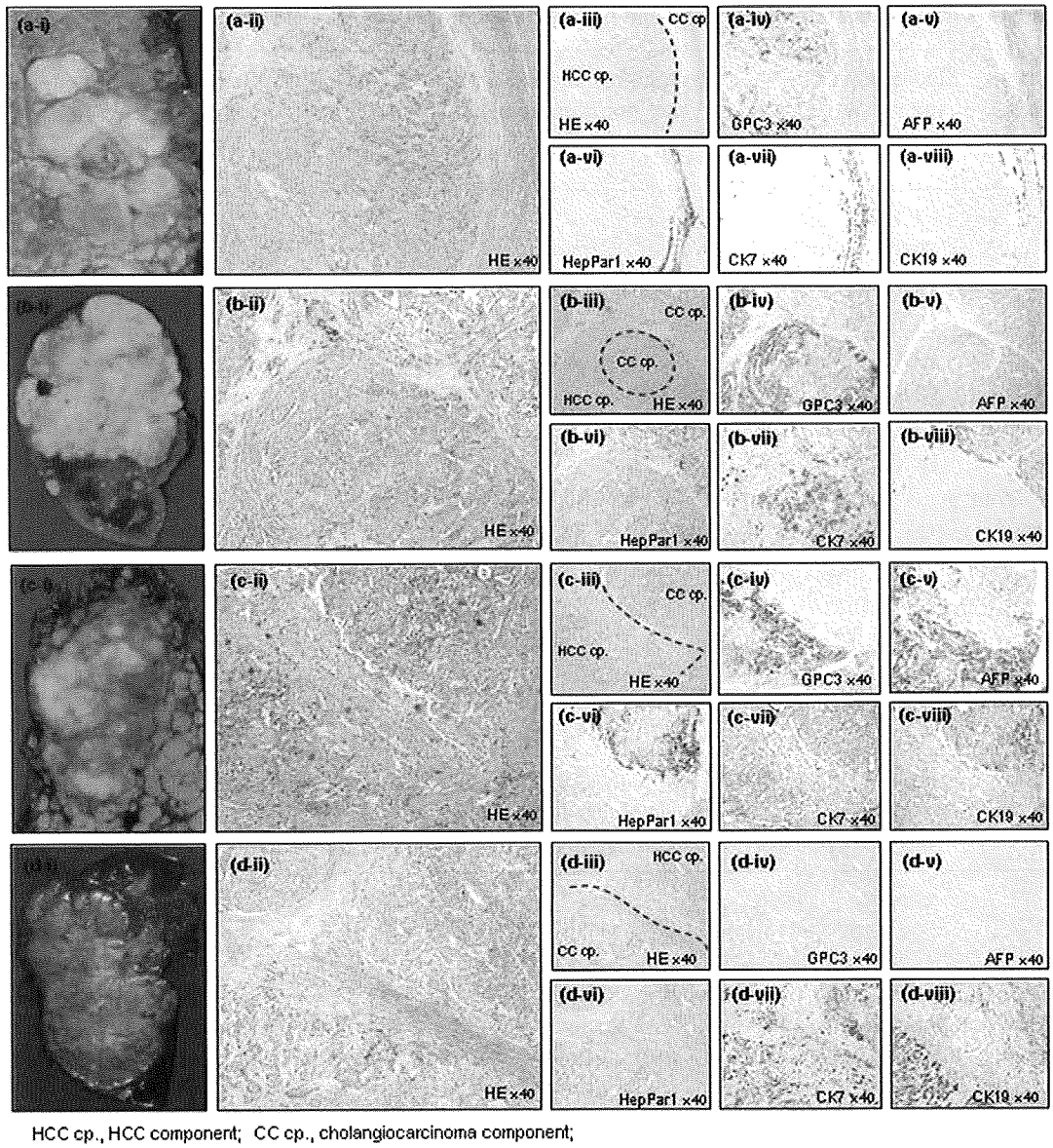

Figure 2. Macroscopic, histological and immunohistochemical features of four cases of CHC, a, case 2; b, case 6; c, case 8; d, case 10 in Table II. (a-i) Macroscopic feature in cut surface of case 2 tumor. (a-ii) The histological structure can be also devided into 2 types. HCC component showed expansive 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.

References

1. Aoki K, Takayasu K, Kawano T, *et al*: Combined hepatocellular carcinoma and cholangiocarcinoma: clinical features and computed tomographic findings. Hepatology 18: 1090-1095, 1993.
2. Ng IO, Shek TW and Nicholls J and Ma LT: Combined hepatocellular-cholangiocarcinoma: a clinicopathological study. J Gastroenterol Hepatol 13: 34-40, 1998.
3. Liu CL, Fan ST, Lo CM, *et al*: Hepatic resection for combined hepatocellular and cholangiocarcinoma. Arch Surg 138: 86-90, 2003.
4. Brumm C, Schulze C, Charels K, Morohoshi T and Kloppel G: The significance of alpha-fetoprotein and other tumour markers in differential immunocytochemistry of primary liver tumours. Histopathology 14: 503-513, 1989.
5. Wennerberg AE, Nalesnik MA and Coleman WB: Hepatocyte paraffin 1: a monoclonal antibody that reacts with hepatocytes and can be used for differential diagnosis of hepatic tumors. Am J Pathol 143: 1050-1054, 1993.
6. Minervini MI, Demetris AJ, Lee RG, Carr BI, Madariaga J and Nalesnik MA: Utilization of hepatocyte-specific antibody in the immunocytochemical evaluation of liver tumors. Mod Pathol 10: 686-692, 1997.
7. Leong AS, Sormunen RT, Tsui WM and Liew CT: Hep Par 1 and selected antibodies in the immunohistological distinction of hepatocellular carcinoma from cholangiocarcinoma, combined tumours and metastatic carcinoma. Histopathology 33: 318-324, 1998.
8. Lau SK, Prakash S, Geller SA and Alsabeh R: Comparative immunohistochemical profile of hepatocellular carcinoma, cholangiocarcinoma, and metastatic adenocarcinoma. Human Pathol 33: 1175-1181, 2002.
9. Maeda T, Kajiyama K, Adachi E, Takenaka K, Sugimachi K and Tsuneyoshi M: The expression of cytokeratins 7, 19, and 20 in primary and metastatic carcinomas of the liver. Mod Pathol 9: 901-909, 1996.
10. Sasaki A, Kawano K, Aramaki M, Nakashima K, Yoshida T and Kitano S: Immunohistochemical expression of cytokeratins in intrahepatic cholangiocarcinoma and metastatic adenocarcinoma of the liver. J Surg Oncol 70: 103-108, 1999.
11. Shimonishi T, Miyazaki K and Nakanuma Y: Cytokeratin profile relates to histological subtypes and intrahepatic location of intrahepatic cholangiocarcinoma and primary sites of metastatic adenocarcinoma of liver. Histopathology 37: 55-63, 2000.
12. Capurro M, Wanless IR, Sherman M, *et al*: Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology 125: 89-97, 2003.
13. Nakatsura T, Yoshitake Y, Senju S, *et al*: Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 306: 16-25, 2003.
14. Sung YK, Hwang SY, Park MK, *et al*: Glypican-3 is overexpressed in human hepatocellular carcinoma. Cancer Sci 94: 259-262, 2003.
15. Hippo Y, Watanabe K, Watanabe A, *et al*: Identification of soluble NH2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. Cancer Res 64: 2418-2423, 2004.
16. Yamauchi N, Watanabe A, Hishinuma M, *et al*: The glypican 3 oncofetal protein is a promising diagnostic marker for hepatocellular carcinoma. Mod Pathol 18: 1591-1598, 2005.
17. Filmus J: The contribution of *in vivo* manipulation of gene expression to the understanding of the function of glypicans. Glycoconj J 19: 319-323, 2002.
18. De Cat B, Muyldermans SY, Coomans C, *et al*: Processing by proprotein convertases is required for glypican-3 modulation of cell survival, Wnt signaling and gastrulation movements. J Cell Biol 163: 625-635, 2003.
19. Capurro MI, Shi W, Sandal S and Filmus J: Processing by convertases is not required for glypican-3-induced stimulation of hepatocellular carcinoma growth. J Biol Chem 280: 41201-41206, 2005.
20. Capurro MI, Xiang YY, Lobe C and Filmus J: Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. Cancer Res 65: 6245-6254, 2005.
21. Song HH, Shi W, Xiang YY and Filmus J: The loss of glypican-3 induces alterations in Wnt signaling. J Biol Chem 280: 2116-2125, 2005.
22. Varma RR, Hector SM, Clark K, Greco WR, Hawthorn L and Pendyala L: Gene expression profiling of a clonal isolate of oxaliplatin-resistant ovarian carcinoma cell line A2780/C10. Oncol Rep 14: 925-932, 2005.
23. Filmus J, Capurro M and Rast J: Glypicans. Genome Biol 9: 224, 2008.
24. Stigliano I, Puricelli L, Filmus J, Sogayar MC, Bal de Kier Joffe E and Peters MG: Glypican-3 regulates migration, adhesion and actin cytoskeleton organization in mammary tumor cells through Wnt signaling modulation. Breast Cancer Res Treat (In press).
25. Torisu Y, Watanabe A, Nonaka A, *et al*: Human homolog of NOTUM, overexpressed in hepatocellular carcinoma, is regulated transcriptionally by beta-catenin/TCF. Cancer Sci 99: 1139-1146, 2008.
26. Jia HL, Ye QH, Qin LX, *et al*: Gene expression profiling reveals potential biomarkers of human hepatocellular carcinoma. Clin Cancer Res 13: 1133-1139, 2007.
27. Nakatsura T, Kageshita T, Ito S, *et al*: Identification of glypican-3 as a novel tumor marker for melanoma. Clin Cancer Res 10: 6612-6621, 2004.
28. Ikuta Y, Nakatsura T, Kageshita T, *et al*: Highly sensitive detection of melanoma at an early stage based on the increased serum secreted protein acidic and rich in cysteine and glypican-3 levels. Clin Cancer Res 11: 8079-8088, 2005.
29. Nakatsura T and Nishimura Y: Usefulness of the novel oncofetal antigen glypican-3 for diagnosis of hepatocellular carcinoma and melanoma. BioDrugs 19: 71-77, 2005.
30. Goodman ZD, Ishak KG, Langloss JM, Sesterhenn IA and Rabin L: Combined hepatocellular-cholangiocarcinoma: a histologic and immunohistochemical study. Cancer 55: 124-135, 1985.
31. Kassahun WT and Hauss J: Management of combined hepatocellular and cholangiocarcinoma. Int J Clin Pract (In press).
32. Taketa K: Alpha-fetoprotein: reevaluation in hepatology. Hepatology 12: 1420-1432, 1990.
33. Tangkijvanich P, Tosukhowong P, Bunyongyod P, *et al*: Alpha-L-fucosidase as a serum marker of hepatocellular carcinoma in Thailand. Southeast Asian J Trop Med Public Health 30: 110-114, 1999.
34. Filmus J and Capurro M: Glypican-3 and alphafetoprotein as diagnostic tests for hepatocellular carcinoma. Mol Diagn 8: 207-212, 2004.
35. Wee A: Fine needle aspiration biopsy of the liver: algorithmic approach and current issues in the diagnosis of hepatocellular carcinoma. Cytojournal 2: 7, 2005.
36. Man XB, Tang L, Zhang BH, *et al*: Upregulation of Glypican-3 expression in hepatocellular carcinoma but downregulation in cholangiocarcinoma indicates its differential diagnosis value in primary liver cancers. Liver Int 25: 962-966, 2005.
37. Tickoo SK, Zee SY, Obiekwe S, *et al*: Combined hepatocellular-cholangiocarcinoma: a histopathologic, immunohistochemical, and *in situ* hybridization study. Am J Surg Pathol 26: 989-997, 2002.
38. Yano Y, Yamamoto J, Kosuge T, *et al*: Combined hepatocellular and cholangiocarcinoma: a clinicopathologic study of 26 resected cases. Jpn J Clin Oncol 33: 283-287, 2003.
39. Nishie A, Yoshimitsu K, Asayama Y, *et al*: Detection of combined hepatocellular and cholangiocarcinomas on enhanced CT: comparison with histologic findings. AJR 184: 1157-1162, 2005.
40. Chu PG, Ishizawa S, Wu E and Weiss LM: Hepatocyte antigen as a marker of hepatocellular carcinoma: an immunohistochemical comparison to carcinoembryonic antigen, CD10, and alpha-fetoprotein. Am J Surg Pathol 26: 978-988, 2002.
41. Zhang F, Chen XP, Zhang W, *et al*: Combined hepatocellular cholangiocarcinoma originating from hepatic progenitor cells: immunohistochemical and double-fluorescence immunostaining evidence. Histopathology 52: 224-232, 2008.
42. Taguchi J, Nakashima O, Tanaka M, Hisaka T, Takazawa T and Kojiro M: A clinicopathological study on combined hepatocellular and cholangiocarcinoma. J Gastroenterol Hepatol 11: 758-764, 1996.
43. Wu PC, Fang JW, Lau VK, Lai CL, Lo CK and Lau JY: Classification of hepatocellular carcinoma according to hepatocellular and biliary differentiation markers. Clinical and biological implications. Am J Pathol 149: 1167-1175, 1996.

44. Nakatsura T, Komori H, Kubo T, *et al*: Mouse homologue of a novel human oncofetal antigen, glypican-3, evokes T-cell-mediated tumor rejection without autoimmune reactions in mice. Clin Cancer Res 10: 8630-8640, 2004.
45. Komori H, Nakatsura T, Senju S, *et al*: Identification of HLA-A2- or HLA-A24-restricted CTL epitopes possibly useful for glypican-3-specific immunotherapy of hepatocellular carcinoma. Clin Cancer Res 12: 2689-2697, 2006.
46. Motomura Y, Senju S, Nakatsura T, *et al*: Embryonic stem cell-derived dendritic cells expressing glypican-3, a recently identified oncofetal antigen, induce protective immunity against highly metastatic mouse melanoma, B16-F10. Cancer Res 66: 2414-2422, 2006.
47. Motomura Y, Ikuta Y, Kuronuma T, *et al*: HLA-A2 and -A24-restricted glypican-3-derived peptide vaccine induces specific CTLs: preclinical study using mice. Int J Oncol 32: 985-990, 2008.
48. Grozdanov PN, Yovchev MI and Dabeva MD: The oncofetal protein glypican-3 is a novel marker of hepatic progenitor/oval cells. Lab Invest 86: 1272-1284, 2006.
49. Durnez A, Verslype C, Nevens F, *et al*: The clinicopathological and prognostic relevance of cytokeratin 7 and 19 expression in hepatocellular carcinoma: a possible progenitor cell origin. Histopathology 49: 138-151, 2006.
50. Komuta M, Spee B, Vander Borght S, *et al*: Clinicopathological study on cholangiolocellular carcinoma suggesting hepatic progenitor cell origin. Hepatology 47: 1544-1556, 2008.
51. Fausto N and Campbell JS: The role of hepatocytes and oval cells in liver regeneration and repopulation. Mech Dev 120: 117-130, 2003.
52. Libbrecht L: Hepatic progenitor cells in human liver tumor development. World J Gastroenterol 12: 6261-6265, 2006.
53. Alison MR, Vig P, Russo F, *et al*: Hepatic stem cells: from inside and outside the liver? Cell Prolif 37: 1-21, 2004.

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

Hirofumi Shirakawa,[1,3] Hitomi Suzuki,[1] Manami Shimomura,[1] Motohiro Kojima,[2] Naoto Gotohda,[3] Shinichiro Takahashi,[3] Toshio Nakagohri,[3] Masaru Konishi,[3] Nobuaki Kobayashi,[4] Taira Kinoshita[3] and Tetsuya Nakatsura[1,5]

[1]Section for Cancer Immunotherapy, Investigative Treatment Division, [2]Pathology Division, Research Center for Innovative Oncology, [3]Hepato-Biliary pancreatic Surgery division, National Cancer Center Hospital East, Chiba; [4]Department of Organ Regulatory Surgery, Ehime University Graduate School of Medicine, Ehime, Japan

(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

[5]To whom correspondence should be addressed. E-mail: tnakatsu@east.ncc.go.jp

doi: 10.1111/j.1349-7006.2009.01206.x

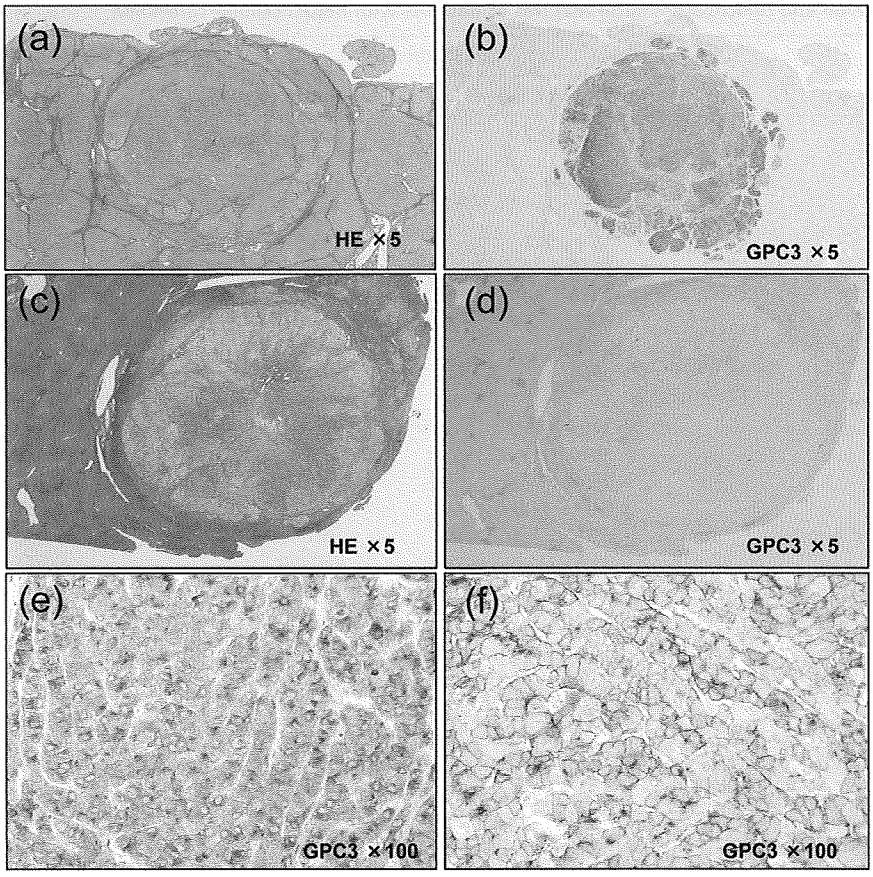

Fig. 1. Glypican (GPC)-3 expression and localization is hepatocellular carcinoma (HCC)-specific. (a,c) Microscopic view of a HE-stained sections of resected HCC. (b,d) HCC sections were stained for GPC3 expression with anti-GPC3 monoclonal antibody. (e) HCC displays prominent bile-canalicular immunostaining.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