表 1　胃癌取扱い規約と治療ガイドラインの役割分担

1．手術を含む治療に関する記述を，ほぼ全面的に規約からガイドラインに移行する
2．原発病変の占居部位別のリンパ節群分類と郭清範囲分類を廃止し，ガイドラインで術式別に郭清範囲を規定する
3．N 分類は，TNM 分類第 6 版の転移個数を採用する
4．術前化学療法の普及を考慮して，リンパ節分類(“L-level”)，進行度分類をあらたに規定する

表 2　胃癌取扱い規約による病期分類

	N0	N1	N2	N3
T1(M)	ⅠA	ⅠB	Ⅱ	Ⅳ
T1(SM)	ⅠA	ⅠB	Ⅱ	Ⅳ
T2(MP)	ⅠB	Ⅱ	ⅢA	Ⅳ
T3(SE)	Ⅱ	ⅢA	ⅢB	Ⅳ
T4(SI)	ⅢA	ⅢB	Ⅳ	Ⅳ
H1, P1, CY1, M1	Ⅳ	Ⅳ	Ⅳ	Ⅳ

の TNM 分類の改定が 2009 年に行われることから，これに対応した規約改定を行い，この新しい規約に対応したガイドラインが作成されることになった．基本方針として，規約は腫瘍の状態(原発病変，転移，進行度)と治療の評価(根治性，薬物の効果)を記録するための基本ルールを示し，その改定は 10 年に一度程度を目安とする．一方，ガイドラインは，手術法を含む各種治療法とその適応など，臨床における具体的な指針を示すとともに，新しいエビデンスに基づいて随時更新し，インターネットで公開することとした．表 1 にいくつか合意された点を列挙したが，手術など治療に関する記述の多くがガイドラインの委員会の役割となった．また，リンパ節分類を個数による TNM 分類を採用することなど，重要な変更も提案された．

TNM分類の改定と取扱い規約

表 2 は現行の日本胃癌学会のステージ分類である．表 3 は第 7 版の TNM 分類であるが，深達度に ss が加わって，より細かい分類となっている．分類は細かくなったが，かつては日本の分類が細かすぎると，欧米から簡便化が主張された時代のあったことを思うと，むしろ従来からの日本の主張が受け入れられるようになったと解釈することもできよう．また，リンパ節に関しては転移個数によって分類している点が，わが国の分類と大きく異なっている点である．周知のようにわが国のリンパ節群分類は解剖学的なリンパ節の位置によって決められてきていたが，一方でわが国のデータをもとに転移個数でステージを分類しても遠隔成績との相関が高いこともよく知られていた．2009 年 3 月の日本胃癌学会総会では，胃癌取扱い規約およびガイドラインについて合同コンセンサスミーティングが 4 時間にわたって行われたが，結論としてはおおむね TNM 分類に沿った病期分類が受け入れられた．今後の改定作業はこのコンセンサスミーティングの結果を尊重した形で進められる予定である．

もともとの TNM 分類改定案は UICC と AJCC (American Joint Committee on Cancer)によって合同でつくられたものであったが，基本的なデータがないために，日本や韓国など胃癌研究の進んだ国にとって受け入れがたいものであった．国際胃癌学会ではこのような状況を憂慮し，日本および韓国のデータを TNM の委員会で提示し強く改正を迫り，それがほぼ受け入れられた形で第 7 版が作成された経緯がある．日本の緻密な検討からはまだ不十分な点があることは否めないが，世界中で日本など胃癌先進国と同様の緻密な分類がそのまま通用するとは考えがたい．今回日本胃癌学会のステージ分類をいったん TNM に添った形にしたうえで，今後の改定の際に科学的なデータの下によりよい分類に替えていくことが混乱を避ける建設的な方法と考えられる．2009 年 6 月にポーランドのクラクフで開催された国際胃癌会議でもこの問題が討議され，今後適切な TNM 分類改定に向けて国際胃癌学会として登録事業などの作業を進めることが承認された．UICC と胃癌のデータのほとんどないアメリカの AJCC が主体となってきた TNM 分類の改定に，国際胃癌学会が積極的に関与することで，今後よりよいものになることが期待される．

新しい規約分類に沿った治療ガイドライン案

新しい規約分類に沿った治療ガイドライン案を表 4 に提示したが，今後，委員会の検討で細部に

表 3　胃癌病期分類(TNM分類，7版)

	NO	N1(1～2)*	N2(2～6)*	N3(7～)*	Any N, M1
T1a-M	ⅠA	ⅠB	ⅡA	ⅡB	Ⅳ
T1b-SM	ⅠA				
T2-MP	ⅠB	ⅡA	ⅡB	ⅢA	
T3-SS	ⅡA	ⅡB	ⅢA	ⅢB	
T4a-SE	ⅡB	ⅢA	ⅢB	ⅢC	
T4b-SI	ⅢB	ⅢB	ⅢC	ⅢC	
Any T, M1	Ⅳ				

(　)*：転移リンパ節個数.

表 4　新しいTNM分類に対応した胃癌治療ガイドライン(案)

	N0	N1(1～2)	N2(3～6)	N3(7～)	Any N, M1
T1a-M	ⅠA ESD(well, m＜2 cm)	ⅠB D1＋No. 8a, 9(＜2.0 cm) D2(＞2.1 cm)	ⅡA D2	ⅡB D2	Ⅳ
T1b-SM	ⅠA D1(well, m＜1.5 cm) D1＋No. 8a, 9				
T2-MP	ⅠB D2	ⅡA D2＋adjuvant chemotherapy	ⅡB D2＋adjuvant chemotherapy	ⅢA D2＋adjuvant chemotherapy	
T3-SS	ⅡA D2＋adjuvant chemotherapy	ⅡB D2＋adjuvant chemotherapy	ⅢA D2＋adjuvant chemotherapy	ⅢB D2＋adjuvant chemotherapy	
T4a-SE	ⅡB D2＋adjuvant chemotherapy	ⅢA D2＋adjuvant chemotherapy	ⅢB D2＋adjuvant chemotherapy	ⅢC D2＋adjuvant chemotherapy	
T4b-SI	ⅢB D2＋adjuvant chemotherapy (combined resection)	ⅢB D2＋adjuvant chemotherapy (combined resection)	ⅢC D2＋adjuvant chemotherapy (combined resection)	ⅢC D2＋adjuvant chemotherapy (combined resection)	
Any T, M1	Ⅳ Chemotherapy, Palliative care, Surgery, Radiation etc.				

ついては変更があるかもしれないので留意されたい．第 2 版が公開された 2004 年以降，化学療法やリンパ節郭清について臨床試験の結果が明らかになり，ガイドラインの速報版で，臨床研究として示されていたもののうちいくつかが日常診療として推奨されるようになった．胃癌治癒切除後の術後補助化学療法は第 2 版でも日常診療ではなく，手術単独が日常診療とされていた．その後，胃癌治癒切除術後の TS-1 投与に関する第Ⅲ相臨床試験が行われ，TS-1 投与群の 3 年生存率が手術単独群に比較して有意に優れていた結果[4]を受けて，すでにガイドラインの速報版で StageⅡ，ⅢA，ⅢB に対する TS-1 による補助化学療法が日常診療として推奨されている．また，臨床研究として行われてきたリンパ節の拡大郭清(3 群，大動脈周囲リンパ節郭清)は，予防的にこれを行っても予後を改善しないことが明らかになったため，行うべきでないとされた．

StageⅣに対する化学療法はガイドラインでも日常診療として記載されているが，現実にどのようなレジメを使うべきかはガイドラインでは示されていなかった．StageⅣに対して化学療法が best supportive care より優れていることを示すエビデンスはいくつかあったものの，いずれも少数例の検討であり，それぞれのレジメも異なっていたため，その時点では特定のレジメを推奨できな

かったのである．

その後，JCOG9912 試験で，切除不能進行・再発胃癌に対して，TS-1 が 5-FU に対して非劣勢であることが示された．そして，SPIRITS 試験では切除不能または再発胃癌に対して CDDP＋TS-1 が，TS-1 より有意に生存期間を延長することが示された結果[5]，現時点では標準的な治療レジメとされている．したがって，これらのレジメは 3 版で日常診療として記載されることになろう．

なお，第 2 版発行以降の進歩については日本胃癌学会が速報版を作成して Web 上で公開しており，日本胃癌学会ホームページを参照していただきたい(http://www.jgca.jp/guideline/index.html)．速報版は，最新の知見をガイドライン委員会でガイドライン同様に評価して，評価委員会と理事会で検討のうえつくられている．また，研究成果は，A：ガイドラインの内容を変更させる重要な成果，B：ガイドラインを変更させるには至らないが，参考にすべき重要な成果，C：ガイドラインに影響を与えない成果，に分けて評価されている．

おわりに

胃癌取扱い規約が純粋に胃癌のステージ決定のための取り決めであることに徹することが合意された結果，そのぶんガイドラインの役割が明確になってきた．これからは胃癌に限らず他の癌についても同様の動きが起こることが予想される．ガイドラインはいまや，各学会の年次総会で徹底的に検討されるべき対象になりつつある．医療の進歩はそれだけ早く，しかも最新の医療の実践が要求されているからである．

ただし，ガイドラインが最善で絶対的に正しい治療方法を示したものであり，あたかもガイドラインを外れた治療が不当で不正なことでもあるかのような受け止め方は間違っている．ガイドラインはあくまでも現時点で，標準的な体力をもった患者にとってもっとも妥当と思われる治療法を提示したものであり，かならずしもそのとおりにしなければならないものではない．もちろん，ガイドラインをまったく無視した医療がまかり通るのは避けるべきであるが，ガイドラインのとおりに行うのが正義という主張も同様に危険であることを認識すべきである．患者全例にガイドラインに沿った医療を提供しているとしたら，その施設は患者個々の病状や事情を無視して治療を行っているに等しい．患者のリスクや社会的背景，病期の微妙な差に注意を払い，ガイドラインを十分に参照しつつ，患者個々に応じた治療法選択し提供できることこそ重要である．ガイドラインと個人個人に対応したいわゆる個別医療とはけっして相対するものではなく，適切な個別医療の実現のためにもガイドラインのような標準的治療の指針が必要なのである．DPC(包括医療)のなかで，ガイドラインを守れば守るほど医療費を上げようなどという暴論が出てくることは，ガイドラインの本質をまったく理解しないものがまだいる証拠である．各学会はガイドラインに対するこのような誤解を無くすように，努力すべきである．

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別添３

厚生労働科学研究費補助金
（総括・分担）研究報告書）

がん診療ガイドラインの作成（新規・更新）と公開の維持およびその在り方に関する研究

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研究要旨
肝癌診療ガイドライン改訂版(2009年版)の公開の手順と維持，外部評価について検討した．改訂版でも紙媒体にて公開しているため，一般公開であるWebでの公開は改訂後1年以上経過しており，より最新の情報提供のためには公開体制の検討が必要と考えられた．ガイドラインの外部評価については，次回改訂時に評価結果がよりよく反映される仕組みが必要であると考えられた．

Ａ．研究目的

肝癌診療ガイドラインは，平成14-15年の厚生労働省ガイドライン支援事業により，2005年初版が発表された．その後，組織を日本肝臓学会に移行し，2009年11月に改訂版が発表された．

本研究では，このガイドラインを広く一般に発信していく公開体制とガイドライン更新に向けた作業の進め方について検討することを目的とした．

Ｂ．研究方法

2009年版肝癌診療ガイドライン改訂後の公開の手順と維持について検討し，肝癌診療ガイドライン初版の公開の手順と維持と比較検討し，問題点について考察した．

さらに改訂版の外部評価と初版の外部評価と比較し，改訂に関する初版外部評価の反映の方法に関する問題点についても検討した．

（倫理面への配慮）

ガイドライン作成や公開に関わる情報のみを取り扱い個人情報は取り扱わないので倫理面について配慮すべき事象はない．

Ｃ．研究結果

（１）肝癌診療ガイドライン改訂版公開について

肝癌診療ガイドラインが初版から4年後に改訂され2009年版として公開された．改訂版では，作製主体が厚生労働研究班から日本肝臓学会に移管され，資金も初版3年6,000万円から3年400万円と大きく減額となった．論文選択の方法も変更され，Evidence-drivenからNecessity-drivenとなり，全文検索からQuestion毎の検索式による検索となった．

改訂版の公開は，まず金原出版からの印刷物での紙媒体を介した公開となった．その後，英語版として肝臓学会の機関誌であるHepatology Researchに6月にSpecial issueとして公開された．Webでの公開については肝臓学会のホームページに2010年10月に公開予定となっているが公開が遅れている．今後，Minds,肝癌研究会，癌治療学会，国立がん研究センターの各ホームページにて公開予定である．

（２）肝癌診療ガイドライン改訂版（2009年度版）外部評価と今後の改訂について

改訂版に関しても外部評価が行われその結果が肝臓学会の機関誌である雑誌「肝臓」に公開された．外部評価は6名で行われ，AGREE(Appraisal of Guidelines for Research and Evaluation) Projectによる評価方法，Shaneyfeltらによる評価方法，COGS(Conference on Guideline Standardization)による評価方法を用いて行われた．AGREEによる評価では6領域の評価項目毎の平均点および標準化スコアは，「対象と目的」3.78点，93%，「利害関係者の参加」2.79点，60%，「作製の厳格さ」3.71点，90%，「明確さと提示の方法」3.33点，78%，「適応可能性」2.56点，52%，「編集の独立性」3.08点，69%であり，2005年版の評価に比して，「明確さと提示の方法」について評価は大きくは変わっていないものの，それ以外の領域についてはいずれも2005年度版の評価を上回っていた．「対象と目的」，「作製の厳格さ」および「明確さと提示の方法」については優れているものの，「利害関係者の参加」及び「適応可能性」についても改善の余地があることが示唆された．以上から改訂版にて初版の外部評価の結果がある程度反映されていることが考えられた．

Ｄ．考察

肝癌診療ガイドライン改訂版の公開については，紙媒体が優先されているため，版権の問題から一般へのWebによる公開は1年以上遅延となっていた．

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Web公開まで1年という期間は版権の問題であり，適正かどうかの判断はされていない．初版同様改訂版でもガイドラインのWeb公開までの遅延が認められている現状および近年の診断治療の急速な進歩に対して，紙媒体でのガイドライン公開自体を見直す必要があるのかもしれない．

ガイドラインの外部評価については，初版の評価に比較しより良好な評価が得られており，初版の評価を反映し作製されたものと考えられた．評価が改善していない「利害関係者の参加」及び「適応可能性」に関しては，肝癌診療ガイドラインがエビデンスとその収集方法を厳格に守って作製されているという特徴によるものも考えられた．

Ｅ．結論

肝癌診療ガイドライン改訂版の公開体制と外部評価の結果から，ガイドラインの公開方法を検討する必要であると考えられた．また，ガイドライン改訂時に前ガイドラインに対する外部評価を反映していくような改訂作業体制の構築も検討する必要がある．

Ｆ．健康危険情報

特記すべきことなし．

Ｇ．研究発表

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Ｈ．知的財産権の出願・登録状況

1. 特許取得

該当事項なし

2. 実用新案登録

該当事項なし

3.その他

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CLINICAL STUDIES

dUTP pyrophosphatase expression correlates with a poor prognosis in hepatocellular carcinoma

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Abbreviations
5 FU, 5 fluorouracil; dUTPase, dUTP pyrophosphatase; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; qRT PCR, quantitative reverse transcription polymerase chain reaction; SAGE, serial analysis of gene expression.

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Abstract

Background: Hepatocellular carcinoma (HCC) is a malignancy with a poor prognosis, partly owing to the lack of biomarkers that support its classification in line with its malignant nature. To discover a novel molecular marker that is related to the efficacy of treatment for HCC and its biological nature, we performed serial analysis of gene expression (SAGE) in HCC, normal liver and cirrhotic liver tissues. *Methods:* Gene expression profiles of HCC tissues and non cancerous liver tissues were obtained by SAGE. Suppression of the target gene by RNA interference was used to evaluate its role in HCC *in vitro*. The relation of the identified marker and prognosis was statistically examined in surgically resected HCC patients. *Results:* We identified significant over expression of *DUT*, which encodes dUTP pyrophosphatase (dUTPase), in HCC tissue, and this was confirmed in about two thirds of the HCC samples by reverse transcription polymerase chain reaction ($n = 20$). Suppression of dUTPase expression using short interfering RNAs inhibited cell proliferation and sensitized HuH7 cells to 5 fluorouracil treatment. Nuclear dUTPase expression was observed in 36.6% of surgically resected HCC samples ($n = 82$) evaluated by immunohistochemistry, and its expression was signifi cantly correlated with the histological grades ($P = 0.0099$). Notably, nuclear dUTPase expression correlated with a poor prognosis with statistical signifi cance (HR, 2.47; 95% CI, 1.08 5.66; $P = 0.032$). *Conclusion:* Taken together, these results suggest that nuclear dUTPase may be a good biomarker for predicting prognosis in HCC patients after surgical resection. Development of novel dUTPase inhibitors may facilitate the eradication of HCC.

Hepatocellular carcinoma (HCC) is the fifth most com mon malignancy and the third leading cause of cancer related death worldwide (1). Several risk factors are responsible for HCC development, including alcoholism, aflatoxin and genetic diseases such as haemochromatosis and α 1 antitrypsin deficiency; however, the major risk factor is chronic hepatitis owing to hepatitis B virus (HBV) or hepatitis C virus (HCV) infection (2 4). Several treatment options are currently available for HCC management, which include liver transplantation, surgical resection, percutaneous ethanol injection, radio frequency ablation, transcatheter arterial chemoemboli zation and systemic or local chemotherapy, and optimal treatment is determined based on tumour stage and liver function (5, 6). However, more than 80% of HCC cases develop advanced HCC after initial treatment (7).

Various chemotherapeutic drugs have been investi gated for their antitumour activity in advanced HCC. For example, 5 fluorouracil (5 FU), a thymidylate synthase inhibitor, was the first reported drug studied for the treatment of advanced HCC; however, a median survival rate of 3 5 months has discouraged the further use of 5 FU as a single chemotherapeutic agent (8, 9). Interferon α (IFN α) has been reported to have antitu mour activity against advanced HCC, and recent reports have suggested the efficacy of a combination of 5 FU/ IFN α for advanced HCC treatment (10 12), although convincing evidence for improved survival rate remains lacking. A recent study has indicated that 16% of advanced HCC patients responded positively to 5 FU/ IFN α treatment with clear and significant survival benefits compared with stable or progressive disease

patients (13). Thus, drug sensitivity appears to be one of the major determinants of the prognosis of advanced HCC patients treated with chemotherapy. Therefore, a hallmark of successful treatment would be the identification of useful biomarkers for determining the survival benefits offered by each treatment strategy.

In this study, we investigated the gene expression profiles of HCCs using serial analysis of gene expression (SAGE) to identify novel molecular markers or targets for the treatment of HCC (14 18). Here, we identified the upregulation of the *DUT* gene that encodes dUTP pyrophosphatase (dUTPase) in HCC. Markedly, HCC with a high nuclear dUTPase expression correlated with a poorly differentiated morphology and a poor prognosis. *DUT* gene knockdown not only suppressed cell proliferation but also sensitized HuH7 cells to low dose 5 FU.

Materials and methods

Samples

All HCC tissues, adjacent non cancerous liver tissues and normal liver tissues were obtained from 110 patients undergoing a hepatectomy between 1997 and 2006 in Kanazawa University Hospital, Kanazawa, Japan. Five normal liver tissue samples were obtained from patients undergoing surgical resection of the liver for the treatment of metastatic colon cancer. These samples were snap frozen in liquid nitrogen immediately after resection. One hundred and five HCC and surrounding non cancerous liver samples were obtained from patients undergoing surgical resection of the liver for HCC treatment, and part of these samples were used for the recent study (19). Three HCC and adjacent non cancerous liver tissue samples were snap frozen in liquid nitrogen and later used for SAGE. Twenty HCC tissues and their corresponding non cancerous liver tissues were also snap frozen and later used for real time reverse transcription polymerase chain reaction (RT PCR) analysis, as described previously (19). Eighty two additional HCC samples were formalin fixed, paraffin embedded and used for immunohistochemistry (IHC). HCC and adjacent non cancerous liver tissues were histologically characterized, as reported elsewhere (19).

All strategies used for gene expression analysis as well as tissue acquisition processes were approved by the Ethics Committee and the Institutional Review Board of Kanazawa University Hospital. All procedures and risks were explained verbally to each patient, who then provided written informed consent.

Serial analysis of gene expression

Total RNA was purified from each homogenized tissue sample using a ToTally RNA extraction kit (Ambion Inc., Austin, TX, USA), and polyadenylated RNA was isolated using a MicroPoly (A) Pure kit (Ambion). A total of 2.5 μg of mRNA per sample was analysed by SAGE (20, 21). SAGE libraries were randomly sequenced at the Genomic Research Center (Shimadzu Biotechnology, Kyoto, Japan), and the sequence files were analysed with SAGE 2000 software. The size of each SAGE library was normalized to 300 000 transcripts per library, and the abundance of transcripts was compared with SAGE 2000 software. Monte Carlo simulation was used for selecting genes whose expression levels were significantly different between the two libraries (22). Each SAGE tag was annotated using a gene mapping website SAGE Genie database (http://cgap.nci.nih.gov/SAGE/) and the Source database (http://smd.stanford.edu/cgi bin/source/sourceSearch), as described previously (23).

Quantitative reverse transcription polymerase chain reaction

A 1 μg aliquot of each total RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real time RT PCR analysis was performed using the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). Using the standard curve method, quantitative PCR was performed in duplicate for each sample primer set. Each sample was normalized relative to β actin. The assay IDs used were Hs00798995 s1 for dUTPase and Hs99999903 m1 for β actin.

RNA interference targeting *DUT*

Small interfering RNAs (siRNAs) targeting *DUT* or control (scrambled sequence) were synthesized by Dharmacon (Dharmacon Research Inc., Lafayette, CO, USA). The target sequences of *DUT* are 5′ AAGUUGUGAAAACGGACAUUC 3′ (DUT1) and 5′ CGGACAUUCAGAUAGCGCUTT 3′ (DUT2). Lipofectamine 2000™ reagent (Invitrogen) was used for transfection according to the manufacturer's instructions.

Cell proliferation assay, soft agar assay and matrigel invasion assay

Cell proliferation assays were performed using a Cell Titer96 Aqueous kit in quintuplicate (Promega, Madison, WI, USA). For the soft agar assay, 1×10^4 cells were suspended in 2 ml of 0.36% agar with growth medium and added in each well of a six well plate containing a base layer of 0.72% agar. The plates were incubated at 37 °C in a 5% CO_2 incubator for 2 weeks. Matrigel invasion assays were performed using BD BioCoat™ Matrigel Matrix Cell Culture Inserts and Control Inserts (BD Biosciences, San Jose, CA, USA), as described in the manufacturer's instruction. 5 FU was obtained from Kyowa Kirin (Kyowa Kirin, Tokyo, Japan). All experiments were repeated at least twice.

Immunohistochemistry

Mouse monoclonal anti dUTPase antibody M01 (Abnova Corporation, Taipei, Taiwan) and mouse antiproliferating

cell nuclear antigen (PCNA) monoclonal antibody PC10 (Calbiochem, San Diego, CA, USA) were used to evaluate the immunoreactivity of HCC and adjacent non cancer ous liver samples using a Dako EnVision+^{TM} kit (Dako, Carpinteria, CA, USA), according to the manufacturer's instruction. Immunoreactivity was evaluated by deter mining the percentage of cells expressing dUTPase in the examined fields, graded as low (0 50%) or high (> 50%). The PCNA index was evaluated as described previously (19).

Statistical analysis

Student's *t* test was used to determine the statistical significance of the differences in cell viability between the two groups. The Mann Whitney *U* test was used for the analysis of gene expression between chronic liver disease (CLD) and HCC tissues. The χ^2 test was used to evaluate the correlation between clinicopathological characteristics and dUTPase expression status. Univari ate and multivariate Cox proportional hazards regression analysis was used to evaluate the association of dUTPase expression and clinicopathological parameters with patient outcome. All statistical analyses were performed using SPSS software (SPSS software package; SPSS Inc., Chicago, IL, USA) and GRAPHPAD PRISM software (Graph Pad Software Inc., La Jolla, CA, USA).

Results

Gene expression profiling identified the overexpression of *DUT* in hepatocellular carcinoma

To overcome the considerable individual variability of transcriptomic characteristics, we constructed a SAGE library of normal human liver using RNAs derived from five normal liver tissues. In addition, we constructed two SAGE libraries derived from three HCC tissues or corre sponding non cancerous liver tissues from patients who developed HCC with a history of chronic hepatitis C. We detected a total of 226 267 tags corresponding to 45 746 unique tags from these SAGE libraries (supporting infor mation Table S1). After excluding the tags detected only once in each library, we selected 15 333 reliable unique transcripts expressed in at least one of the SAGE libraries to avoid contamination of tags derived from sequence errors. Then, we annotated these transcripts using SAGE Genie database and the Source database to identify the potential subcellular localization of transcripts categorized into eight groups in each SAGE library.

The number of nuclear component related tran scripts was increased in the HCC library compared with the normal liver and non cancerous liver libraries, whereas the other cellular component related tran scripts did not show this tendency (supporting infor mation Fig. S1). Because nuclear component related genes may closely correlate with cancer cell prolifera tion and chemosensitivity (24), we further investigated the expression of nuclear component related tags in each library, and identified 10 transcripts associated with nucleotide/nucleoside metabolism that are over expressed in HCC (Table 1). Using Monte Carlo simu lation, we evaluated the significance of differentially expressed transcripts in HCC and corresponding CLD libraries or in HCC and normal liver libraries. We identified a *DUT* gene encoding dUTPase (dUTPase) whose expression was significantly altered ($P = 0.01$). We also identified a *TS* gene encoding thymidylate synthase in the list, but the difference did not reach statistical significance.

dUTPase is a phosphatase known to maintain a dUMP pool by catalysing the hydrolysis of dUTP to dUMP, and thus provides a substrate of thymidylate synthase. Its role in HCC is unknown; therefore, we examined *DUT* expression in 20 independent HCC and corresponding non cancerous liver tissues, and identified significant overexpression of *DUT* in HCC tissue ($P = 0.0015$) (Fig. 1A). Moreover, we detected more than a two fold in crease in *DUT* expression in 70% of HBV related and HCV related HCC cases (14 of 20 HCCs) compared with the non cancerous liver tissues (Fig. 1B). We further examined the expression of *DUT* in 238 HCC tissues compared with the non cancerous liver tissues using publicly available microarray data (GSE5975) (Fig. S2). Consistent with the SAGE data, *DUT* was overexpressed more than two fold in 121 of 238 HCC tissues (median: 2.03), whereas *TS* was overexpressed more than two fold in 54 of 238 HCC tissues (median: 1.41) compared with the non cancerous liver tissues.

Pivotal role of dUTP pyrophosphatase expression in cell proliferation in hepatocellular carcinoma cell lines

In general, cancer gene signatures discovered by compar ison between tumour and non tumour tissues are more likely to reflect the differences in the control of cell proliferation and growth (25). Accordingly, we investi gated the function of dUTPase in cell proliferation in HuH7 cells by *DUT* gene knockdown. *DUT* expression was decreased by 60 70% following the transfection of the siRNA constructs specifically targeting *DUT* 48 h after transfection (DUT1 in Fig. 2A and DUT2 in Fig. S3A), and cell growth was significantly inhibited com pared with the control 72 h after transfection (Fig. 2B and Fig. S3B). Anchorage independent cell growth was also significantly impaired by *DUT* gene knockdown 14 days after transfection (Fig. 2C). Furthermore, *DUT* gene knockdown decreased the numbers of both migrating and invading cells 72 h after transfection (Fig. 2D and E).

dUTPase is known to be associated with thymidylate synthesis (26), and thus we evaluated the effects of 5 FU, a thymidylate synthase inhibitor, on dUTPase expression in HCC cell lines *in vitro*. When we treated HuH7 cells with low dose 5 FU (0.25 mg/ml), we could not detect any growth inhibitory effects (Fig. 2F). Based on this condition, we evaluated the effect of *DUT* gene knock down on 5 FU sensitivity 72 h after transfection.

Table 1. Genes associated with nucleic acid metabolism overexpressed in hepatocellular carcinoma

Tag sequence	Normal liver	Non cancerous liver	HCC	Fold*	Gene	*P* value†
CAGCTCCGCT	0	2	11	5.5	dUTP pyrophosphatase	0.010
AAAGGATAAT	0	0	3	> 3	General transcription factor II H, polypeptide 2	0.127
ACGGTCCAGG	0	0	3	> 3	Cytidine deaminase	0.127
ATGTAGAGTG	0	0	3	> 3	Thymidylate synthase	0.127
TGGGGATTAC	1	0	3	> 3	Zinc ribbon domain containing, 1	0.127
CACCCTGTAC	2	2	6	3	Solute carrier family 29	0.147
GAACGCCTAA	1	1	3	3	Dihydropyrimidinase like 2	0.308
GCGCTGGTAC	0	1	3	3	2′ 5′ oligoadenylate synthetase 3	0.308
CTTAGTGCAA	0	2	4	2	3′ phosphoadenosine 5′ phosphosulphate synthase 2	0.335
TTGTTACATC	0	2	3	1.5	Phosphoribosyl pyrophosphatase synthetase associated protein 1	0.506

*Fold increase was calculated by dividing the number of tags in HCC by that of tags in non cancerous liver. To avoid division by 0, a tag value of 1 was used for any tag that was not detectable in one sample.

†Statistical significance of differentially expressed genes between two groups (HCC and non cancerous liver libraries) was calculated using Monte Carlo simulation.

HCC, hepatocellular carcinoma.

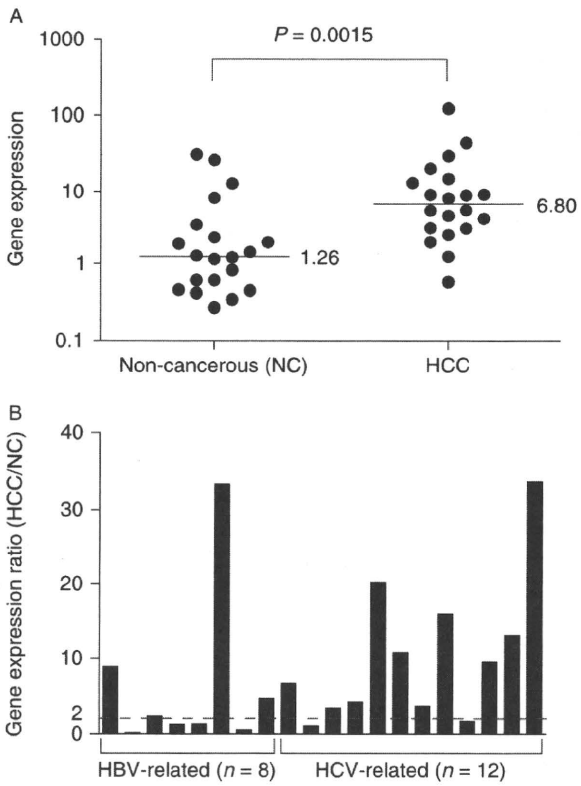

Fig. 1. (A) Quantitative reverse transcription polymerase chain reaction analysis of *DUT* expression in hepatocellular carcinoma (HCC) and corresponding non cancerous liver tissues. *DUT* was significantly activated in HCC tissues compared with non cancerous liver tissues ($P = 0.0015$). A median value in each group is indicated. (B) *DUT* gene expression ratios of HCC and corresponding non cancerous liver tissues. Fourteen of 20 HCC tissues expressed *DUT* more than two fold compared with the background non cancerous liver tissues. HBV, hepatitis B virus; HCV, hepatitis C virus.

Interestingly, *DUT* gene knockdown not only suppressed cell proliferation but also sensitized HuH7 cells to low dose 5 FU (Fig. 2F and Fig. S3B). These data suggest that dUTPase overexpression in HCC tissues may be associated with enhanced cell proliferation and 5 FU resistance.

Intense dUTP pyrophosphatase expression is correlated with a poor prognosis in hepatocellular carcinoma patients

To characterize the clinicopathological characteristics of dUTPase expression in HCC, we performed IHC using an additional independent HCC cohort. Accordingly, we explored the dUTPase expression in HCC using 82 formalin fixed paraffin embedded HCC specimens. All HCC tissues were surgically resected at the Liver Disease Center of Kanazawa University Hospital with full clinical information, and their immunoreactivity to anti dUTPase antibodies was evaluated by IHC. We noticed that anti dUTPase antibodies reacted to both nuclear (red arrows) and cytoplasmic (blue arrows) isoforms of dUTPase, as described previously (26) (Fig. 3A and B). We therefore evaluated the nuclear and cytoplasmic expression of dUTPase separately. We stratified HCC tissues and evaluated the dUTPase expression status based on the percentages of dUTPase positive cells. The frequency of nuclear or cytoplasmic dUTPase positive cells was highly variable in each HCC tissue, and we defined HCCs with nuclear or cytoplasmic dUTPase expressed in $\geq 50\%$ of tumour cells as nuclear or cytoplasmic dUTPase high HCC (Fig. 3C). Nuclear dUTPase overexpression was detected in 36.6% (30 of 82), whereas cytoplasmic dUTPase overexpression was detected in 67.1% (55 of 82) of HCC tissues compared with the corresponding non cancerous liver tissues

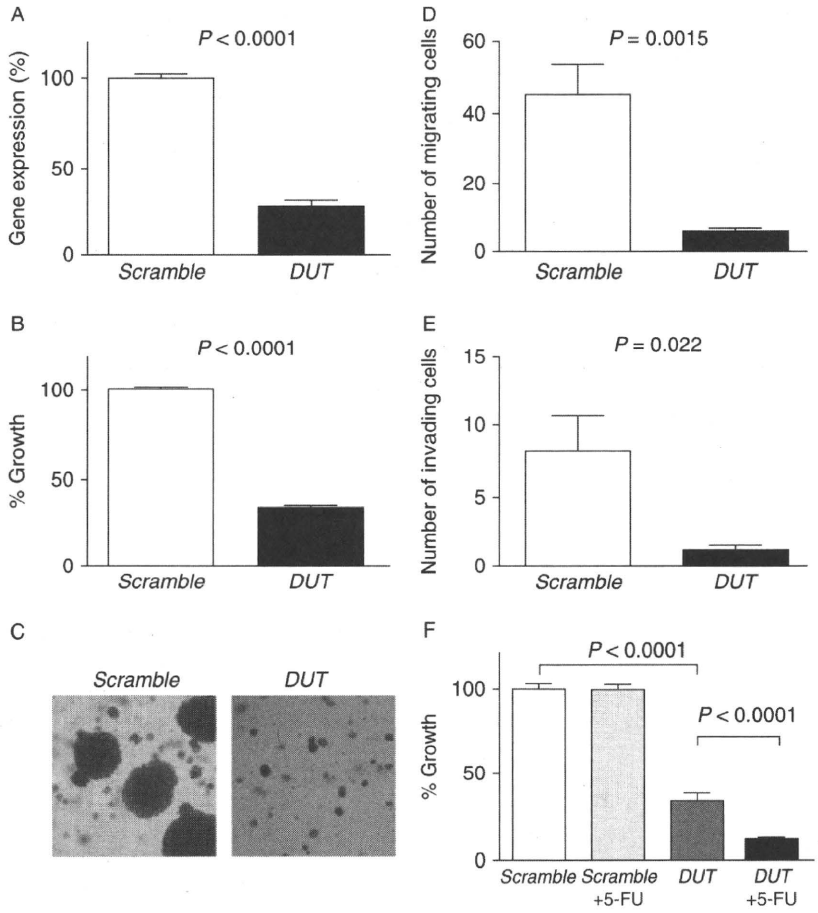

Fig. 2. (A) Transfection of small interfering RNAs targeting *DUT* (DUT1) decreased *DUT* expression compared with the control (scrambled sequence). Gene expression was evaluated in triplicate 72 h after transfection (mean ± SD). (B) *DUT* gene knockdown significantly suppressed cell proliferation ($P < 0.0001$). Cell viability was evaluated in triplicate 72 h after transfection (mean ± SD). (C) Soft agar assay. *DUT* gene knockdown suppressed anchorage independent cell growth. (D and E) Matrigel invasion assay. *DUT* gene knockdown decreased the numbers of both migrating and invading cells. Experiments were performed in triplicate (mean ± SD). (F) *DUT* gene knockdown sensitized HuH7 cells to low dose 5 fluorouracil (5 FU) (0.25 μg/ml), which had no effect on the cell proliferation in the control (mean ± SD).

(Table 2). In general, non cancerous hepatocytes rarely expressed nuclear dUTPase (Fig. 3A).

We investigated the clinicopathological characteristics of nuclear or cytoplasmic dUTPase in low /high HCC cases (Table 2). The expression status of nuclear dUTPase showed no correlation with age, gender, virus, presence of cirrhosis, α fetoprotein value, tumour size and TNM stages. However, nuclear dUTPase expression was signifi cantly correlated with the histological grades of HCC ($P = 0.0099$), and high frequencies of nuclear dUTPase positive cells were associated with poorly differentiated cell morphology in the HCC tissue. In contrast, cyto plasmic dUTPase expression was not correlated with the histological grades of HCC ($P = 0.077$). We examined the cell proliferation of these HCC samples by PCNA stain ing, and PCNA indexes were significantly higher in nuclear dUTPase high HCC than low HCC with statis tical significance ($P = 0.01$) (Fig. S4).

We further investigated the prognostic significance of dUTPase expression in HCC. Strikingly, high nuclear dUTPase expression in HCC tissue correlated with a poor survival outcome compared with low nuclear dUTPase expression ($P = 0.0036$), whereas high cytoplas mic dUTPase expression had little effects when evaluated by recurrence free survival (Fig. 3D). Furthermore, uni variate Cox regression analysis showed a significant correlation between high nuclear dUTPase expression and a high risk of mortality (HR, 2.47; 95% CI, 1.08 5.66; $P = 0.032$; Table 3). By multivariate Cox regression analysis, TNM stage (HR, 2.75; 95% CI, 1.11 6.79; $P = 0.027$) and nuclear dUTPase (HR, 2.61; 95% CI, 1.13 6.05; $P = 0.024$) were independent prog nostic factors associated with a high risk of mortality, and other clinicopathological features did not add indepen dent prognostic information. These data indicate a sig nificant correlation between the malignant potential of

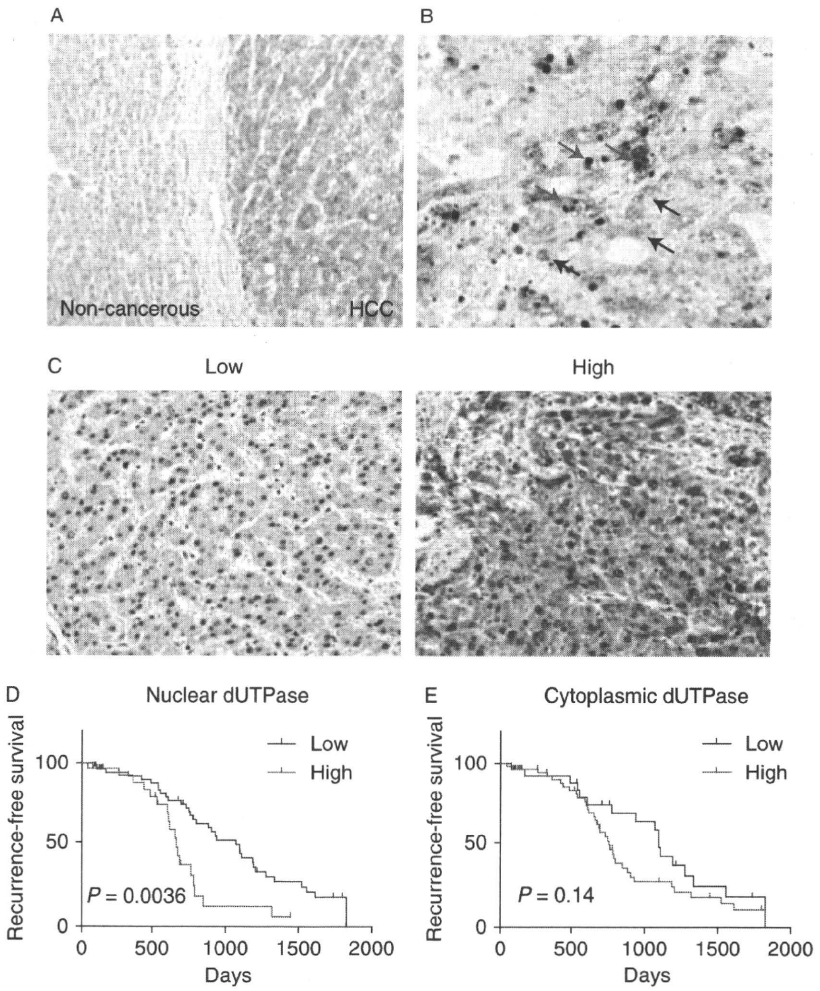

Fig. 3. Immunohistochemistry analysis of dUTP pyrophosphatase (dUTPase) expression in hepatocellular carcinoma (HCC). (A) A representative photomicrograph of dUTPase staining in an HCC and adjacent non cancerous liver tissue. (B) A representative photomicrograph of dUTPase staining in an HCC. Both nuclear (red arrows) and cytoplasmic (blue arrows) forms of dUTPase were detected. (C) Representative photomicrographs of HCC tissues with low (0 50%) and high (≥50%) frequencies of nuclear and cytoplasmic dUTPase positive cells. (D and E) Kaplan Meier survival analysis of HCC tissues with nuclear (D) or cytoplasmic (E) dUTPase expression. High percentages of nuclear dUTPase positive tumour cells significantly correlated with poor clinical outcome in recurrence free survival.

HCC and nuclear dUTPase expression, implicating the potential effectiveness of nuclear dUTPase level as a biomarker for predicting the survival of HCC patients after surgical resection.

Discussion

Here, using a global gene expression profiling approach (18), we have identified the activation of the nucleotide/nucleoside metabolism related gene *DUT* (encoding dUTPase) in HCC. Notably, an intense dUTPase expression was detected in a subset of HCC with a poor prognosis. To the best of our knowledge, this is the first report describing the correlation between dUTPase activation and poor survival outcome in HCC patients.

In normal cells, dUTPase is known to catalyse the hydrolysis of dUTP to dUMP in order to maintain the dUMP pool at a certain level for thymidylate synthesis (26). Interestingly, dUTPase mutations in *Escherichia coli* increased dUTP levels, leading to dUTP misincorporation into DNA during replication, which resulted in DNA fragmentation and apoptosis (27). Furthermore, introduction of *E. coli* dUTPase into human tumour cells resulted in the induction of resistance to fluorodeoxyuridine cytotoxicity (28), suggesting a pivotal role of dUTPase in the prevention of DNA damage. Thus, dUTPase activation in the nucleus appears to be critical

Table 2. Clinicopathological characteristics and dUTP pyrophosphatase expression in hepatocellular carcinoma ($n = 82$)

dUTPase expression (nuclear)	Low ($n = 52$)	High ($n = 30$)	*P* value
Age (< 60 years/≥ 60 years)	19/33	8/22	0.36
Sex (male/female)	36/16	23/7	0.47
Virus (HBV/HCV/B+C/NBNC)	15/33/1/3	10/20/0/0	0.48
Cirrhosis (yes/no)	33/19	22/8	0.36
AFP (< 20 ng/ml/≥ 20 ng/ml)	32/20	15/15	0.31
Histological grade*			
I II	14	3	
II III	36	20	
III IV	2	7	0.0099
Tumour size (< 3 cm/≥ 3 cm)	31/21	19/11	0.74
TNM classification† (I, II/III, IV)	43/9	25/5	0.94
dUTPase expression (cytoplasmic)	Low ($n = 27$)	High ($n = 55$)	*P* value
Age (< 60 years/≥ 60 years)	10/17	17/38	0.58
Sex (male/female)	19/8	40/15	0.82
Virus (HBV/HCV/B+C/NBNC)	8/17/1/1	17/36/0/2	0.56
Cirrhosis (yes/no)	17/10	38/17	0.58
AFP (< 20 ng/ml/≥ 20 ng/ml)	16/11	31/24	0.80
Histological grade*			
I II	7	10	
II III	20	36	
III IV	0	9	0.077
Tumour size (< 3cm/≥ 3 cm)	17/10	33/22	0.80
TNM classification† (I, II/III, IV)	21/6	47/8	0.39

*Edmondson Steiner grades.
†UICC TNM classification of liver cancer, 6th edition (2002).
AFP, α fetoprotein; dUTPase, dUTP pyrophosphatase; HBV, hepatitis B virus; HCV, hepatitis C virus.

Table 3. Cox regression analysis of recurrence free survival rate relative to dUTP pyrophosphatase expression and clinicopathological parameters ($n = 82$)

	Univariate		Multivariate	
Variables (n)	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Child Pugh				
A	1			
B	1.73 (0.50 5.97)	0.38		
Tumour size				
< 3 cm ($n = 50$)	1			
≥ 3 cm ($n = 32$)	1.58 (0.69 3.63)	0.28		
TNM stage*				
I, II ($n = 68$)	1		1	
III, IV ($n = 14$)	2.57 (1.05 6.29)	0.039	2.75 (1.11 6.79)	0.027
Serum AFP				
< 20 ng/ml ($n = 49$)	1			
≥ 20 ng/ml ($n = 38$)	1.54 (0.66 3.56)	0.31		
Microvascular invasion				
No	1			
Yes	1.98 (0.89 4.44)	0.095		
BCLC stage				
A	1			
B/C	2.16 (0.93 5.00)	0.07		
Cytoplasmic dUTPase				
Low ($n = 27$)	1			
High ($n = 55$)	1.15 (0.50 2.62)	0.73		
Nuclear dUTPase				
Low ($n = 52$)	1		1	
High ($n = 30$)	2.47 (1.08 5.66)	0.032	2.61 (1.13 6.05)	0.024

*UICC TNM classification of liver cancer, 6th edition (2002).
AFP, α fetoprotein; CI, confidence intervals; dUTPase, dUTP pyrophosphatase; HR, hazard ratio.

for preventing DNA damage possibly at the S phase. Specifically, this activation may prevent dUTP misincorporation in various cancers and thus avert DNA damage and apoptosis induction. Indeed, dUTPase activation has recently been reported in colorectal and brain cancer (29, 30), and dUTPase accumulation might correlate with 5 FU based chemotherapy resistance and poor prognosis in colorectal cancer (26).

If dUTPase activation plays a central role in the development of resistance to thymidylate synthase inhibitors in order to prevent a DNA damage response, dUTPase inhibition may facilitate the eradication of cancer cells by sensitizing these cells to such inhibitors. Indeed, a recent study suggested a drastic sensitization of colon cancer cells to 5 FU by siRNAs mediated dUTPase suppression (31, 32), which is consistent with our current observation. Because all HCC samples used in this study were surgically resected, we could not evaluate the effect of dUTPase expression on clinical HCC patients' outcome in relation to chemosensitivity to thymidylate synthase inhibitors. Nevertheless, intense nuclear dUTPase expression may be a good biomarker for predicting the response to thymidylate synthase inhibitors, and its usefulness should be further evaluated in the future.

In conclusion, comprehensive gene expression profiling shed new light on the role of dUTPase in HCC. Nuclear dUTPase accumulation is potentially a good biomarker for predicting poor prognosis in HCC patients, and the development of a dUTPase inhibitor may promote the possibility of tumour eradication in HCC patients.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Subcellular localization of genes detected in each SAGE library.

Fig. S2. Microarray analysis of *DUT* and *TS* gene expression in 238 HCC cases publicly available (GSE5975). *DUT* was overexpressed more than 2 fold in 121 of 238 HCC tissues (median: 2.03), whereas *TS* was overexpressed more than 2 fold in 54 of 238 HCC tissues (median: 1.41) compared with the non cancerous liver tissues.

Fig. S3. (A) Transfection of siRNAs targeting *DUT* (DUT2) decreased *DUT* expression compared with the control (scrambled sequence). Gene expression was eval uated in triplicates 72 hours after transfection (mean ± SD). (B) *DUT* gene knockdown sensitized HuH7 cells to low dose 5 FU (0.25 mg/ml) (mean ± SD).

Fig. S4. Nuclear and cytoplasmic dUTPase expres sion and cell proliferation in HCC. PCNA indexes in nuclear dUTPase high HCC were higher than those in low HCC with statistical significance ($P = 0.01$). Cyto plasmic dUTPase expression was not associated with PCNA indexes in HCC.

Table S1. A summary of constructed SAGE libraries.

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AACR

Cancer Research

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Tumor and Stem Cell Biology

Cancer Research

Oncostatin M Renders Epithelial Cell Adhesion Molecule–Positive Liver Cancer Stem Cells Sensitive to 5-Fluorouracil by Inducing Hepatocytic Differentiation

Taro Yamashita, Masao Honda, Kouki Nio, Yasunari Nakamoto, Tatsuya Yamashita, Hiroyuki Takamura, Takashi Tani, Yoh Zen, and Shuichi Kaneko

Abstract

Recent evidence suggests that a certain type of hepatocellular carcinoma (HCC) is hierarchically organized by a subset of cells with stem cell features (cancer stem cells; CSC). Although normal stem cells and CSCs are considered to share similar self renewal programs, it remains unclear whether differentiation programs are also maintained in CSCs and effectively used for tumor eradication. In this study, we investigated the effect of oncostatin M (OSM), an interleukin 6 related cytokine known to induce the differentiation of hepatoblasts into hepatocytes, on liver CSCs. OSM receptor expression was detected in the majority of epithelial cell adhe sion molecule positive ($EpCAM^+$) HCC with stem/progenitor cell features. OSM treatment resulted in the induction of hepatocytic differentiation of $EpCAM^+$ HCC cells by inducing signal transducer and activator of transcription 3 activation, as determined by a decrease in stemness related gene expression, a decrease in EpCAM, α fetoprotein and cytokeratin 19 protein expressions, and an increase in albumin protein expression. OSM treated $EpCAM^+$ HCC cells showed enhanced cell proliferation with expansion of the EpCAM negative non CSC population. Noticeably, combination of OSM treatment with the chemotherapeutic agent 5 fluorouracil (5 FU), which eradicates EpCAM negative non CSCs, dramatically increased the number of apoptotic cells *in vitro* and suppressed tumor growth *in vivo* compared with either saline control, OSM, or 5 FU treatment alone. Taken together, our data suggest that OSM could be effectively used for the differentiation and active cell division of dormant $EpCAM^+$ liver CSCs, and the combination of OSM and conventional chemotherapy with 5 FU efficiently eliminates HCC by targeting both CSCs and non CSCs. *Cancer Res; 70(11); 4687 97. ©2010 AACR.*

Introduction

It is widely accepted that cancer is a disease that develops from a normal cell with accumulated genetic/epigenetic changes. Although considered monoclonal in origin, cancer is composed of heterogeneous cellular populations. These heterogeneities are traditionally explained by the clonal evo lution of cancer cells through a series of stochastic genetic events (clonal evolution model; ref. 1). In contrast, cancer cells are known to have the capabilities characteristic of stem cells with respect to self renewal, limitless division, and gen eration of heterogeneous cell populations. Recent evidence suggests that tumor cells possess stem cell features (cancer stem cells; CSC) to self renew and give rise to relatively dif ferentiated cells through asymmetric division, and thereby form heterogeneous populations (CSC model; refs. 2, 3). Accumulating evidence supports the notion that CSCs could generate tumors more efficiently in immunodeficient mice than non CSCs in the case of leukemia and various solid tumors (4 9), although the origin of CSCs is still a contro versial issue.

Worldwide, hepatocellular carcinoma (HCC) is one of the most common malignancies with poor outcome (10). Recent evidence suggests that at least some HCCs are organized by liver CSCs in a hierarchical manner (11). Several markers have been identified as useful for the enrichment of liver CSCs, including side population fraction (12), CD133 (13), CD90 (14), and OV6 (15). We have recently used epithelial cell adhesion molecule (EpCAM) and α fetoprotein (AFP) to identify novel prognostic HCC subtypes related to certain developmental stages of human liver lineages (16). Among these, EpCAM positive ($^+$) AFP^+ HCC (hepatic stem cell like HCC) is characterized by young onset of disease, activation of Wnt/β catenin signaling, and poor prognosis. *EPCAM* is a target gene of Wnt/β catenin signaling (17), and we previ ously identified that $EpCAM^+$ HCC cells from primary HCC

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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samples and cell lines have the features of CSCs, at least in the hepatic stem cell like HCC subtype (18). Thus, EpCAM seems to be a potentially useful marker for the isolation of liver CSCs in hepatic stem cell like HCC.

CSCs are considered to be resistant to chemotherapy and radiotherapy (19 21), which may be associated with the recurrence of the tumor after treatment. These findings have led to the proposal of "destemming" CSCs, to induce the dif ferentiation of CSCs into non CSCs or to eradicate CSCs by inhibiting the signaling pathway responsible for self renewal (22). Recent studies support this proposal and suggest the utility of bone morphogenetic proteins, activated during embryogenesis and required for differentiation of neuronal stem cells, to induce differentiation of brain CSCs and facil itate brain tumor eradication (23, 24). However, it is still debatable whether simple differentiation of CSCs effectively eradicates tumors (25).

Oncostatin M (OSM), an interleukin (IL) 6 related cyto kine produced by $CD45^+$ hematopoietic cells, is known to enhance hepatocytic differentiation of hepatoblasts by induc ing the activation of the signal transducer and activator of transcription 3 (STAT3) pathway (26). Although OSM, IL 6, and leukemia inhibitory factor share STAT3 signaling cas cades, OSM is known to exploit the distinct hepatocytic dif ferentiation signaling in an OSM receptor (OSMR) specific manner (27). In this study, we hypothesized that OSM induces hepatocytic differentiation of liver CSCs through the OSMR signaling pathway. We examined OSMR expres sion and the effect of OSM in $EpCAM^+$ HCC in terms of hepatocytic differentiation and antitumor activities.

Materials and Methods

Clinical HCC specimens

A total of 107 HCC tissues and adjacent noncancerous liver tissues were obtained from patients who underwent hepatectomy for HCC treatment from 1999 to 2007 in Kana zawa University Hospital. These samples were formalin fixed and paraffin embedded, and used for immunohistochemis try. HCC and adjacent noncancerous liver tissues were histo logically diagnosed by two pathologists. An additional fresh $EpCAM^+$ AFP^+ HCC sample was obtained from a surgically resected specimen and immediately used for the preparation of single cell suspensions and xenotransplantation. All tissue acquisition procedures were approved by the Ethics Commit tee and the Institutional Review Board of Kanazawa University Hospital. All patients provided written informed consent.

Cell culture and reagents

HuH1 and HuH7 cells were cultured as previously described (18). A primary HCC tissue was dissected and di gested in 1 μg/mL of type 4 collagenase (Sigma Aldrich Japan K.K.) solution at 37°C for 15 to 30 minutes. Contaminated RBC were lysed with ammonium chloride solution (STEM CELL Technologies) on ice for 5 minutes. $CD45^+$ leukocytes and Annexin V^+ apoptotic cells were removed by autoMACS pro cell separator and magnet beads (Miltenyi Biotec K.K.). EpCAM positive and negative cells were enriched by auto MACS pro cell separator and CD326 (EpCAM) MicroBeads (Miltenyi Biotec K.K.). Recombinant OSM was purchased from R&D Systems, Inc. 5 Fluorouracil (5 FU) was obtained from Kyowa Kirin.

Quantitative reverse transcription PCR analysis

Total RNA was extracted using TRIzol (Invitrogen) accord ing to the instructions of the manufacturer. The expression of selected genes was determined in triplicate using the 7900 Sequence Detection System (Applied Biosystems). Each sample was normalized relative to β actin expression. Probes used were *TACSTD1*, Hs00158980_m1; *AFP*, Hs00173490_m1; *KRT19*, Hs00761767_s1; *hTERT*, Hs00162669_m1; *Bmi1*, Hs00180411_m1; *POU5F1*, Hs00999632_g1; *CYP3A4*, Hs00430021_m1; *OSMR*, Hs00384278_m1; and *ACTB*, Hs99999903_m1 (Applied Biosystems).

Western blotting

Whole cell lysates were prepared using radioimmuno precipitation assay lysis buffer as described previously (28). Rabbit polyclonal antibodies to STAT3 (Cell Signaling Technology, Inc.), rabbit polyclonal anti OSMR antibodies H 200 (Santa Cruz Biotechnology), mouse monoclonal anti phosphorylated STAT3 (Tyr^{705}) antibody (3E2; Cell Signaling Technology), and mouse monoclonal anti β actin antibody (Sigma Aldrich) were used. Immune complexes were visual ized by enhanced chemiluminescence (Amersham Biosciences, Corp.) as described by the manufacturer.

Immunohistochemistry and immunofluorescence analyses

Immunohistochemistry was performed using Envision+ kits (DAKO) according to the instructions of the manufac turer. Anti EpCAM monoclonal antibody, VU 1D9 (Oncogene Research Products), was used for detecting EpCAM. Goat anti OSMR polyclonal antibodies (C 20) were obtained from Santa Cruz Biotechnology. Mouse anti CYP3A4 polyclonal antibodies (Abnova), mouse anti cytokeratin (CK) 19 mono clonal antibody (DAKO), and mouse anti Ki 67 monoclonal antibody MIB 1 (DAKO) were used for detecting CYP3A4, CK19, and Ki 67, respectively. Samples with >5% positive staining in a given area for a particular antibody were considered to be positive. For immunofluorescence analyses, anti EpCAM antibody (Oncogene Research Products), anti gp130ST antibodies (Santa Cruz Biotechnology), and anti phosphorylated STAT3 (Tyr^{705}) antibody (3E2; Cell Sig naling Technology) were used. Alexa 488 FITC conjugated anti mouse IgG or Alexa 568 Texas red conjugated anti goat/rabbit IgG (Molecular Probes) were used as secondary antibodies. Confocal fluorescence microscopic analysis was performed essentially as previously described (18).

Fluorescence activated cell sorting analyses

Cultured cells were trypsinized, washed, and resuspended in HBSS (Lonza) supplemented with 1% HEPES and 2% fetal bovine serum (FBS). Cells were then incubated with FITC conjugated anti EpCAM monoclonal antibody Clone Ber EP4 (DAKO) on ice for 30 minutes, and analyzed using

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a FACSCalibur (BD Biosciences). Intracellular AFP, CK19, and albumin levels were examined using a BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences), anti AFP mouse monoclonal antibody (Nichirei Biosciences Inc.), anti CK19 mouse monoclonal antibody (DAKO), and rabbit polyclonal anti albumin antibodies (Cell Signaling Technology), respectively.

Cell proliferation and colony formation assay

For cell proliferation assays, 2×10^3 cells were seeded in 96 well plates and cultured with 1% FBS DMEM (control), 1% DMEM with OSM (100 ng/mL), 5 FU (2 μg/mL), or OSM (100 ng/mL) and 5 FU (2 μg/mL) for 3 to 7 days without media changes. Cell viability was evaluated in quadruplicate using a CellTiter 96 AQueous kit (Promega). For colony formation assays, 1×10^3 cells were harvested in a one well Culture Slide (BD Biosciences) and cultured with 1% FBS DMEM (control) with or without OSM (100 ng/mL). Culture medium was replaced every 3 days and the colonies were fixed with ice cold 100% methanol and used for immunofluorescence 10 days after the initiation of treatment.

RNA interference

SiRNAs specific to OSMR (Silencer Select siRNA S17542) and a control siRNA (Silencer Select Negative Control no. 1) were obtained from Ambion (Applied Biosystems). To each well of a six well plate, 2×10^5 cells were seeded 12 hours before transfection. Transfection was performed using LipofectAMINE 2000 (Invitrogen), according to the instructions of the manufacturer. A total of 100 pmol/L of siRNA duplex was used for each transfection.

Apoptosis assay

Cells were cultured in 1% FBS DMEM (control), 1% FBS DMEM with OSM (100 ng/mL), 5 FU (2 μg/mL), or OSM (100 ng/mL) and 5 FU (2 μg/mL) for 3 days in six well plates or in culture slides (BD Biosciences). Annexin V binding to cell membranes was visualized using Annexin V FITC antibodies and a FACSCalibur flow cytometer (BD Biosciences). Activation of caspase 3 was visualized by immunohistochemistry or immunofluorescence using anti active caspase 3 polyclonal antibodies (Promega), as described by the manufacturer.

Animal studies

Six week old NOD/SCID mice (NOD/NCrCRl *Prkdc*^{scid}) were purchased from Charles River Laboratories, Inc. The protocol was approved by the Kanazawa University Animal Care and Use Committee. One million tumor cells were suspended in 200 μL of DMEM and Matrigel (1:1), and a s.c. injection was performed. The incidence and size of subcutaneous tumors were recorded. Intratumoral injections of 50 μL of PBS (control), OSM (2 μg/tumor), 5 FU (250 μg/tumor), or OSM (2 μg/tumor) and 5 FU (250 μg/tumor) were initiated twice weekly 48 days after the injection of tumor cells when the average volume of four tumors in each group had reached 400 mm^3. For histologic evaluation, tumors were formalin fixed and paraffin embedded.

Statistical analyses

The association of OSMR expression and clinicopathologic characteristics in HCC was examined using either Mann Whitney *U* or χ^2 tests. Student's *t* test was used to compare various test groups assayed by quantitative reverse transcription PCR analysis. All analyses were performed using Graph Pad Prism software.

Results

Distinct expression of OSMRs in HCC

Before exploring the effect of OSM on HCC, we examined the expression of its receptor, OSMR, in surgically resected HCC and adjacent noncancerous liver tissues by immunohistochemistry. Representative staining of OSMRs in tumor/nontumor tissues is shown in Fig. 1A. In general, cell surface and cytoplasmic immunoreactivity to OSMR were rarely detected in hepatocytes in chronic hepatitis liver (a), but were frequently detected in small hepatocyte like cells in the stroma or transitional cells in the lobule of cirrhotic liver (b), as indicated by the arrows. Note that immunoreactivity to OSMR was not detected in bile duct epithelia or ductular reactions in which EpCAM$^+$ hepatic progenitor cells are thought to accumulate (Supplementary Fig. S1), suggesting that OSMRs might be expressed in hepatic progenitor cells committed to hepatocytes. Immunoreactivity to OSMRs was more strongly detected in HCC than in noncancerous liver (c), and the expression was heterogeneous in the tumor. Of note, OSMRs were detected in HCC cells at the invasive front area of the tumor (d) where CSCs are known to invade frequently (arrows).

Immunoreactivity to OSMR antibodies and EpCAM antibodies was detected in 66 (61.7%) and 38 (35.5%) of 107 HCC specimens, respectively. The clinicopathologic characteristics of OSMR$^+$ and OSMR$^-$ HCC cases are shown in Table 1. OSMR$^+$ HCC was characterized by high serum AFP values ($P = 0.009$), poorly differentiated morphology ($P < 0.0001$), and a high frequency of EpCAM$^+$ HCCs ($P = 0.024$), suggesting that the OSMR is expressed in HCC with stem/progenitor cell features. OSMR$^+$ HCC was also characterized by young onset of disease and male dominance, although these features did not reach statistical significance ($P = 0.052$ and 0.058, respectively). OSMR was more frequently detected in EpCAM$^+$ HCCs (76.3%) than in EpCAM$^-$ HCCs (53.7%). Expression of OSMR and EpCAM was further investigated by double immunofluorescence analysis, and immunoreactivity to OSMR was detected in both EpCAM$^+$ normal hepatic progenitors (Fig. 1B) and EpCAM$^+$ HCC cells (Fig. 1C). These data suggest that although OSMR is more widely expressed than EpCAM in HCC, OSMR is frequently expressed in EpCAM$^+$ normal hepatic progenitors and liver CSCs.

OSM induces hepatocytic differentiation of EpCAM+ HCC

Because OSMR was expressed in the majority of EpCAM$^+$ HCCs, we investigated the effect of OSM on EpCAM$^+$ HCC cell lines. First, we examined the expression of OSMR and its signal transducer glycoprotein 130 (gp130) in EpCAM$^+$ AFP$^+$ HCC cell lines HuH1 and HuH7 by immunofluorescence

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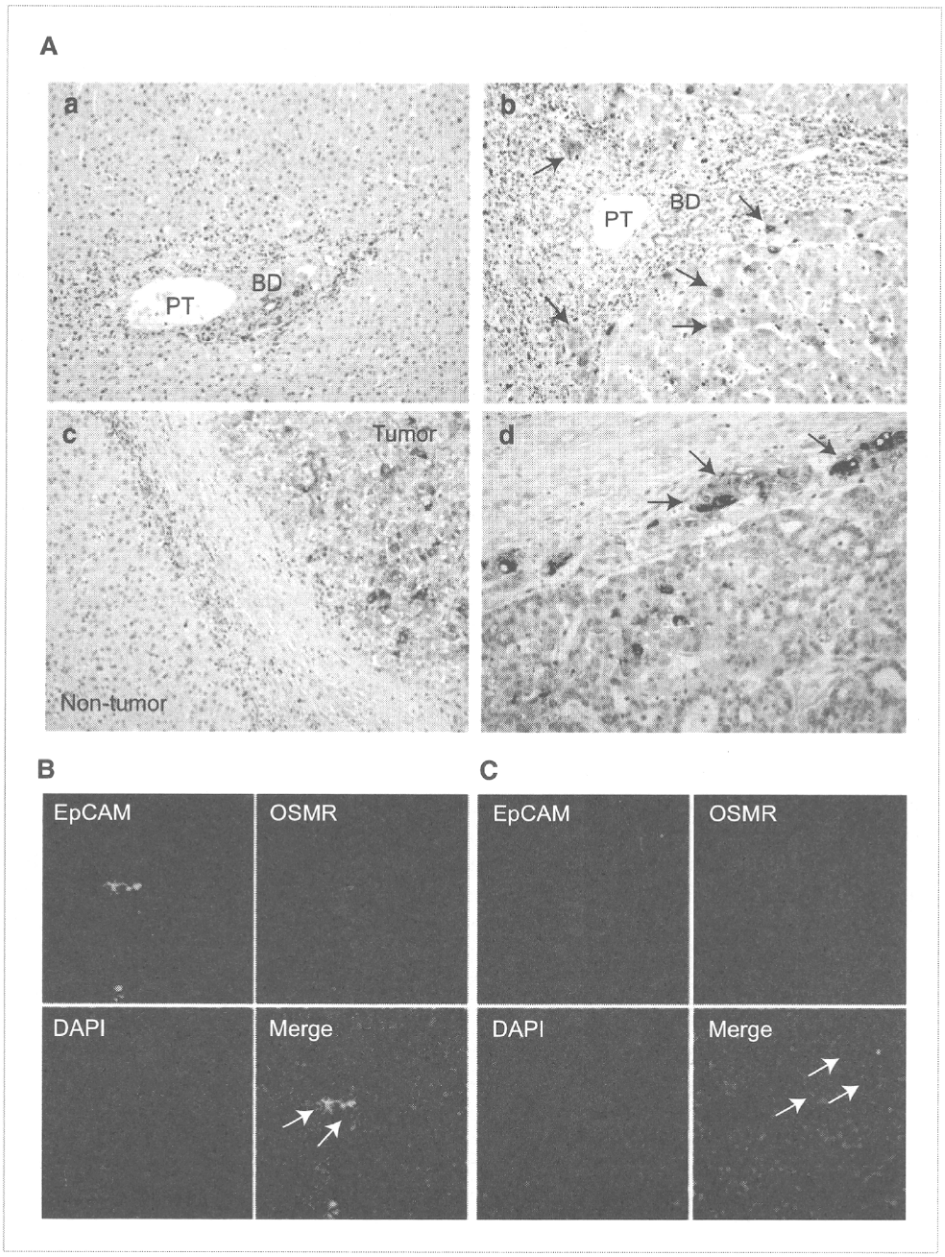

Figure 1. A, representative images of OSMR staining in noncancerous liver tissues and HCC tissues. Immunoreactivity to OSMR was not detected in hepatocytes in chronic hepatitis liver tissue (a) but was detected in a subset of small hepatocyte like cells in the stroma or transitional cells in the lobule (b, arrows) of cirrhotic liver tissue. OSMR was more abundantly expressed in HCC than in noncancerous liver (c). OSMR$^+$ cancer cells were disseminated in the invasive front area of the tumor (d, arrows). PT, portal tract; BD, bile duct. B and C, double immunofluorescence analysis of EpCAM (green) and OSMR (red) expression in noncancerous (B) and HCC (C) tissues.

(Fig. 2A). Both gp130 and OSMR protein expressions were detected in these cells, consistent with the immunohisto chemical data. Because OSM is known to induce the hepato cytic differentiation of hepatoblasts in a STAT3 dependent manner, we investigated the effect of OSM on phosphoryla tion of STAT3 in HuH1 and HuH7 cells by immunofluores cence and Western blotting. Incubation of HCC cells for 1 hour with OSM at a concentration of 100 ng/mL resulted in the induction and nuclear accumulation of phosphorylated STAT3 compared with controls (Fig. 2B and C). We examined the effect of OSM on the EpCAM$^+$ cell population in HuH1 and HuH7 cells. We first labeled HuH1 and HuH7 cells with CD326 (EpCAM) MicroBeads and FITC conjugated anti EpCAM antibodies (Clone Ber EP4) and performed positive/negative selection using magnetic activated cell sorting to determine the appropriate gating criteria for EpCAM high (designated as EpCAM$^+$) and EpCAM low/negative (designated as EpCAM$^-$) cell population (Fig. 2D, top). It is interesting that OSM treatment (100 ng/mL for 72 hours) diminished the EpCAM$^+$ cell population from 50.7% to 10.1% in HuH1 and from 55.2% to 28.8% in HuH7 cells when the same constant gating criteria was applied (Fig. 2D, bottom).

We used RNA interference to investigate whether the decrease in EpCAM$^+$ cells by OSM treatment depends on the expression of OSMR. Transfection of siRNAs specific to *OSMR* (si OSMR) resulted in the knockdown of target genes

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compared with the control (si Control) in HuH1 and HuH7 cells 48 hours after transfection (Supplementary Fig. S2A). We further confirmed the decrease of OSMR protein expression by immunofluorescence and Western blotting 72 hours after transfection (Supplementary Fig. S2B and C). When we treated these HuH1 and HuH7 cells with OSM (100 ng/mL) for 1 hour, we observed the decrease of phos phorylated STAT3 by *OSMR* gene silencing compared with the control (Supplementary Fig. S2C). Furthermore, OSM mediated decrease in the number of EpCAM$^+$ cells was inhibited by *OSMR* gene silencing (Supplementary Fig. S2D), suggesting that OSM exploits the diminution of EpCAM$^+$ cells through the activation of the OSMR signaling pathway in EpCAM$^+$ HCC.

We further examined the effect of OSM on hepatocytic dif ferentiation by quantitative reverse transcription PCR and fluorescence activated cell sorting (FACS) analyses. OSM treatment in HuH1 cells reduced the expression of hepatic progenitor related genes including *AFP, KRT19* (encoding CK19), and *TERT* (encoding telomerase reverse transcriptase; TERT; Fig. 3A). OSM treatment further reduced the expres sion of *BMI1* and *POU5F1* (encoding Oct4), which is known to be expressed and required for self renewal in embryonic stem cells. OSM treatment also increased the expression of the hepatocyte marker, *CYP3A4*. Furthermore, OSM treat ment reduced AFP$^+$ and CK19$^+$ cells and increased albumin$^+$ cells compared with the untreated controls, as evaluated by the geometric mean of the fluorescence intensities of whole cells analyzed by intracellular FACS (Fig. 3B). Similar results were obtained in HuH7 cells (data not shown) and, taken together, these data suggest that OSM induced the hepatocytic differentiation of EpCAM$^+$ HCCs.

Hepatocytic differentiation of EpCAM+ HCC by OSM augments cell proliferation

In general, normal stem cells are more quiescent than dif ferentiated cells in terms of cell division. We therefore eval uated the effect of OSM on cell proliferation in HuH1 and HuH7 cells. It is interesting that OSM treatment for 10 days resulted in a larger colony formation following treatment with OSM (100 ng/mL) compared with untreated controls. Of note, the majority of cells comprising these larger colo nies were EpCAM$^-$, or had low expression levels, whereas a subset of untreated control cells maintained high EpCAM expression (Fig. 3C). Similar results were obtained when cell proliferation was examined using a [3 (4, 5 dimethylthiazol 2 yl) 5 (3 carboxymethoxyphenyl) 2 (4 sulfophenyl) 2H tetrazolium] tetrazolium assay and Ki 67 labeling index (Fig. 3D). OSM modestly enhanced cell proliferation (top) and increased Ki 67 positive cells (middle and bottom) compared with un treated controls in both HuH1 and HuH7 cells with statistical significance (Fig. 3D).

OSM treatment increases chemosensitivity of EpCAM+ HCC

The abovementioned data imply that although OSM may induce the hepatocytic differentiation of dormant EpCAM$^+$ liver CSCs, OSM treatment alone might instead enhance cell prolif eration through expansion of amplifying differentiated cancer cells *in vitro*, raising the question of efficacy of differentiation therapy in EpCAM$^+$ HCC. Because rapidly amplifying cells are considered to be more sensitive to chemotherapeutic agents, we investigated the effect of combining OSM treatment with conventional chemotherapy to target both dormant CSCs and amplifying non CSCs. We have shown that 5 FU treatment

Table 1. Clinicopathologic characteristics of OSMR$^+$ and OSMR$^-$ HCC cases used for immunohisto-chemical analyses

Variables	OSMR$^+$ (n = 66)	OSMR$^-$ (n = 41)	P*
Age (years, mean ± SE)	62.7 ± 1.3	66.4 ± 1.3	0.052
Sex (male/female)	55/11	27/14	0.058
Etiology (HBV/HCV/other)	25/35/6	8/30/3	0.10
Liver cirrhosis (yes/no)	43/23	26/15	1.0
AFP (ng/mL, mean ± SE)	6,453 ± 5901	1,039 ± 935	0.009
Histologic grade†			
I–II	3	16	
II–III	54	20	
III–IV	9	5	<0.0001
Tumor size (<3 cm/>3 cm)	30/36	15/26	0.42
Tumor-node-metastasis classification			
I/II	48	31	
III/IV	18	10	0.82
EpCAM (positive/negative)	29/37	9/32	0.024

*Mann-Whitney U test or χ^2 test.
†Edmondson-Steiner.