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●原 著●

脂溶性白金錯体SM-11355（ミリプラチン水和物）による肝動脈化学塞栓療法

—塞栓材併用時の安全性と有効性について—

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Transcatheter Arterial Chemoembolization with a Lipophilic Platinum Complex SM-11355 (Miriplatin Hydrate)—Safety and Efficacy in Combination with Embolizing Agents: Kenji Ikeda[*1], Takuji Okusaka[*2], Masafumi Ikeda[*3] and Manabu Morimoto[*4] (*[1]*Dept. of Hepatology, Toranomon Hospital, *[2]*Hepatobiliary and Pancreatic Oncology Division, National Cancer Center Hospital, *[3]*Division of Hepatobiliary and Pancreatic Oncology, National Cancer Center Hospital East, *[4]*Gastroenterological Center, Yokohama City University Medical Center*)

Summary

SM-11355 is a cisplatin derivative with high affinity for iodized ethyl esters of fatty acids of poppyseed oil. Clinical trials have shown that SM-11355 is effective for treatment of hepatocellular carcinoma. Transcatheter arterial chemoembolization is commonly used in combination with embolizing agents, but concomitant use of SM-11355 and embolizing agents has not been evaluated in previous trials. In this study, the safety and efficacy of SM-11355 in combination with embolizing agents were investigated in 10 patients with hepatocellular carcinoma. An anti-tumor effect of TE4 was achieved in 4 of 9 patients and no serious adverse events were observed, indicating that this therapy can be used safely for hepatocellular carcinoma. **Key words:** Hepatocellular carcinoma, Miriplatin, Transcatheter arterial chemoembolization (*Received Oct. 2, 2009/Accepted Nov. 16, 2009*)

要旨 SM-11355はcisplatinの誘導体であり，ヨード化ケシ油脂肪酸エチルエステルへの親和性が高く，これまでに実施した臨床試験で肝細胞癌の治療に用いる薬剤としての有用性が示唆されている。一般に肝動脈化学塞栓療法では塞栓物質を併用する方法が選択されているが，これまでに実施したSM-11355の臨床試験では塞栓物質の併用経験がない。そこで今回，10名の肝細胞癌患者を対象に，塞栓物質を併用した際の安全性および有効性について検討した。抗腫瘍効果は9名中4名でTE 4が得られ，重篤な有害事象は認められず，肝細胞癌の治療を行う上で大きな問題は認められなかった。

はじめに

SM-11355（一般名:ミリプラチン水和物）は，国立がんセンター研究所の前田らにより見いだされたcisplatinの誘導体であり，ヨード化ケシ油脂肪酸エチルエステルへの親和性が高く，抗腫瘍効果も良好であると報告されている[1]。cisplatinは最も強力な抗癌剤の一つとして現在でも広く使用されている[2]が，ヨード化ケシ油脂肪酸エチルエステルへの懸濁性が悪く，肝動脈化学塞栓療法(transcatheter arterial chemoembolization: TACE)に用いる抗癌剤としての評価は定まっていない。SM-11355は，cisplatinと同様の細胞増殖抑制活性を有し，ヨード化ケシ油脂肪酸エチルエステルへの懸濁性に優れ，ヨード化ケシ油脂肪酸エチルエステルとともに腫瘍局所に滞留して白金成分を持続的に放出するという特性を有している。この特性により，抗腫瘍効果を持続するとともに，全身循環への移行が微量であることから，全身性の副作用の軽減が可能と考え，肝動脈化学塞栓療法

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に用いる抗癌剤として開発中である。

本剤は1994年に第Ⅰ相臨床試験[3]を開始し，2005年までに前期第Ⅱ相臨床試験[4]，後期第Ⅱ相臨床試験および継続投与試験の計4試験を実施した。後期第Ⅱ相臨床試験では，本剤は対照薬であるzinostatin stimalamerと同程度の抗腫瘍効果を示した。また，zinostatin stimalamerでの発現が知られている肝動脈の血管障害[5-7]は認められず，特に安全性に問題がないことから，肝細胞癌の治療に用いる薬剤としての有用性が示された。

TACEを行う上で一般に塞栓物質を併用すると，その血流遮断効果から併用しない場合に比べ抗腫瘍効果が高まるとされており[8]，多くの場合で塞栓物質を併用する方法が選択されている[9]。しかし，これまでに実施した臨床試験では本剤と塞栓物質との併用経験がないことから，本治験では少数の肝細胞癌患者を対象に，塞栓物質（多孔性ゼラチン粒: 以下塞栓材）を併用した場合の安全性および有効性を検討した。

Ⅰ．対象および方法

本治験では，肝切除術，経皮的エタノール注入療法（percutaneous ethanol injection therapy: PEIT），経皮的マイクロ波凝固療法（percutaneous microwave coagulation therapy: PMCT）またはラジオ波凝固療法（radiofrequency ablation therapy: RFA）の適応外の患者で，以下の基準を満たす患者を対象とした。なお，前治療として肝切除術，PEIT，PMCTまたはRFAを施行した場合は，施行後4週間以上，TACEを施行した場合は，施行後3か月以上経過した患者を対象とした。

主な選択基準

1）組織学的または臨床的（血管造影，CTなど）に肝細胞癌と診断され，測定可能病変（造影CTで最長径が10 mm以上の病変）を有する患者

2）進行度分類（Stage）がⅡまたはⅢの患者

3）Child-Pugh分類がAまたはBの患者

4）肝障害度（liver damage）がAまたはBの患者

5）performance status（PS）が0，1，2の患者

6）同意取得日の年齢が20歳以上，75歳未満の患者

7）文書による同意を得られた患者

また，主な除外基準として胆管内，門脈内または肝静脈内に腫瘍栓，血栓のある患者，治験薬投与直前の血管造影時に以下の基準を満たした患者は除外した。

1）選択的なカテーテル挿入のための適切な肝動脈の構造を有さない患者，または腫瘍濃染像を認めない患者

2）塞栓材の併用が不適切と判断された患者

3）治療領域に肝内シャントを認める患者

4）前治療がある場合は，前治療部位に生残腫瘍を認める患者

表1 患者背景

登録患者数	10名
性別（男性：女性）	8名：2名
年齢（中央値）	67.5歳
Child-Pugh分類（A：B）	7名：3名
初発・再発（初発：再発）	7名：3名
腫瘍数（1個：2個：3個：≧4個）	6名：2名：1名：1名
最大腫瘍径（中央値）（範囲）	36.5 mm（22～65 mm）
Stage（Ⅱ：Ⅲ）	5名：5名

投与液はSM-11355注にヨード化ケシ油脂肪酸エチルエステルを注入し，直ちに（1分間以内）液中に明らかな塊がなくなるまでよく振り混ぜて懸濁し，濃度が20 mg/mLとなるように調製した。

調製したSM-11355懸濁液をX線透視下に固有肝動脈より可能な限り末梢から投与し，その後非イオン性造影剤と混和した塞栓材を注入した。投与液量の上限は6 mL（ミリプラチンとして120 mg），塞栓材の総使用量は1バイアル（多孔性ゼラチン粒として80 mg）以内とした。なお，塞栓材は粒径1 mm規格または2 mm規格のいずれかを使用した。

投与回数は原則1回とし，条件により2回目投与も可能とした。なお，2回目投与時の塞栓材の併用は患者の状態などにより適宜判断することとし，必須とはしなかった。

抗腫瘍効果の判定には，直接治療効果度（TE）「日本肝癌研究会 肝癌治療直接効果判定基準（2004年改訂版）」を判定基準として用いた。各患者の直接治療効果度判定は，最終投与後5週または12週あるいは中止時の画像診断の最大効果により評価した。

安全性の評価には「Common Terminology Criteria for Adverse Events v3.0（CTCAE v3.0）JCOG/JSCO版」を判定基準として用いた。

Ⅱ．結　果

1．患者背景および投与状況

2007年10月～2008年2月までに10名が登録された。年齢の中央値は67.5歳（47～73歳），Child-Pugh分類の内訳はAが7名，Bが3名，StageはⅡおよびⅢがそれぞれ5名であった（表1）。10名すべてに1回目投与が行われ，そのうち3名に2回目投与が行われた。投与回当たりの投与液量の中央値は4.0 mL（1.1～6.0 mL）で，1回目および2回目のいずれの投与時も塞栓材が併用された。治験中止となったのは2名で，その内訳は除外基準に抵触していたことが判明した患者が1名（投与後に肝内シャントが認められていたことが判明），原疾

表 2 抗腫瘍効果（直接治療効果度）

	評価患者数	TE4	TE3	TE2	TE1	TE4の割合（%）[95%信頼区間]
直接治療効果度	9	4	3	1	1	44.4 [13.7～78.8]

抗腫瘍効果の判定には，直接治療効果度（TE）「日本肝癌研究会 肝癌治療直接効果判定基準（2004年改訂版）」を用いた。

表 3 腫瘍マーカーの推移

<1回目投与>

患者番号	TE	AFP (ng/mL) 投与前		AFP (ng/mL) 投与後5週		AFP (ng/mL) 投与後12週		PIVKA-II (mAU/mL) 投与前		PIVKA-II (mAU/mL) 投与後5週		PIVKA-II (mAU/mL) 投与後12週	
01	4	22	H	7		3		65	H	21		18	
02	3	26	H	29	H	33	H	94	H	29		28	
03	2	7		7		9		16,400	H	5,680	H	10,300	H
04	1	8.7		6.9		—		1,077	H	8,427	H	—	
05	4	35.3	H	32.1	H	32.8	H	34		63	H	39	
06	2	111.2	H	83.8	H	177.9*	H	54	H	38		26*	
07	4	8		5		5		67	H	53	H	61	H
08	3	81	H	29	H	34	H	14		10		20	
10	3	12.9	H	11.7	H	14.2	H	263	H	18		23	

TE: 抗腫瘍効果
H: 実施医療機関の基準値上限より高値
*: 投与後62日目のデータ

<2回目投与>

患者番号	TE	AFP (ng/mL) 投与前		AFP (ng/mL) 投与後5週		AFP (ng/mL) 投与後12週		PIVKA-II (mAU/mL) 投与前		PIVKA-II (mAU/mL) 投与後5週		PIVKA-II (mAU/mL) 投与後12週	
03	2	9		7		7		10,300	H	7,070	H	7,190	H
06	3	177.9	H	105.2	H	100.6	H	26		40	H	30	
08	4	34	H	23	H	33	H	20		19		18	

TE: 抗腫瘍効果
H: 実施医療機関の基準値上限より高値

患の悪化により後治療が必要となった患者が1名で，いずれも1回目投与後に治験中止となった。投与された10名すべてを安全性評価の対象とし，1回目投与後に除外基準に抵触していたことが判明した1名を除く9名を有効性評価の対象とした。

2．抗腫瘍効果

9名中4名がTE 4であった（表2）。

3．腫瘍マーカーの推移

AFPでは1回目投与および2回目投与とも投与前後で大きな違いは認められなかった。PIVKA-IIでは7名が投与前に異常値を示していたが，1回目投与後5週で4名の患者が正常化した。また，2回目投与では投与前後で大きな違いは認められなかった（表3）。

4．安全性

本治験では，死亡した患者，重篤な有害事象，有害事象により投与を中止した患者は認められなかった。grade別では，grade 4および5の有害事象は認められなかった。grade 3の有害事象は，AST増加が4名，ALT増加，血中ブドウ糖増加，血小板数減少およびリンパ球数減少がそれぞれ2名，失神が1名に発現した（表4，5）。失神は原疾患に対する追加治療の必要性を聞いたことによる精神的なショックによるものであった。これらはすべて無処置で経過観察され，リンパ球数減少，AST増加のそれぞれ1名を除き回復した。未回復のリンパ球数減少およびAST増加も投与2週後までにgrade 1となった。なお，2回目投与を行った患者では，grade 3以上の有害事象は発現せず，2回目投与によるgradeの悪化は認められなかった。

Ⅲ．考　察

SM-11355に塞栓材を併用した場合の安全性および有効性を，肝細胞癌患者10名で検討した。

表 4 主な有害事象（grade 別）: 血液学的検査

	評価患者数: 10 名						
	grade						合計
	1	2	3	4	5	3≦	
ヘマトクリット減少	9	0	0	0	0	0	9
好酸球百分率増加	7	1	0	0	0	0	8
血小板数減少	2	3	2	0	0	2	7
ヘモグロビン減少	7	0	0	0	0	0	7
単球百分率増加	6	0	0	0	0	0	6
白血球数減少	2	3	0	0	0	0	5
赤血球数減少	5	0	0	0	0	0	5
好中球数減少	1	3	0	0	0	0	4
リンパ球数減少	0	1	2	0	0	2	3

grade 3 以上が発現または 4 名以上に発現した有害事象について発現例数を記載
安全性の評価には「Common Terminology Criteria for Adverse Events v3.0（CTCAE v3.0）JCOG/JSCO 版」を用いた。

表 5 主な有害事象（grade 別）: 非血液学的検査

	評価患者数: 10 名						
	grade						合計
	1	2	3	4	5	3≦	
C-反応性蛋白増加	9	1	0	0	0	0	10
ALT 増加	2	5	2	0	0	2	9
尿中クレアチニン減少	9	0	0	0	0	0	9
AST 増加	0	4	4	0	0	4	8
血中アルブミン減少	2	6	0	0	0	0	8
β-N アセチル D グルコサミニダーゼ増加	7	1	0	0	0	0	8
血中ビリルビン増加	5	2	0	0	0	0	7
血中乳酸脱水素酵素増加	7	0	0	0	0	0	7
血中ブドウ糖増加	4	0	2	0	0	2	6
血中ナトリウム減少	6	0	0	0	0	0	6
尿中クレアチニン増加	5	0	0	0	0	0	5
血中アミラーゼ増加	3	1	0	0	0	0	4
尿中蛋白陽性	3	1	0	0	0	0	4
総蛋白増加	4	0	0	0	0	0	4
血圧上昇	4	0	0	0	0	0	4
発熱	2	8	0	0	0	0	10
食欲不振	8	0	0	0	0	0	8
投与部位疼痛	5	1	0	0	0	0	6
悪心	6	0	0	0	0	0	6
失神	0	0	1	0	0	1	1

grade 3 以上が発現または 4 名以上に発現した有害事象について発現例数を記載
安全性の評価には「Common Terminology Criteria for Adverse Events v3.0（CTCAE v3.0）JCOG/JSCO 版」を用いた。

抗腫瘍効果（直接治療効果度）としては，「日本肝癌研究会 肝癌治療直接効果判定基準（2004年改訂版）」を用いて判定した結果，9名中4名がTE 4であり，塞栓材併用による抗腫瘍効果の増強が期待された。

安全性では10名での評価結果ではあるが，死亡を含む重篤な有害事象や中止に至る有害事象の発現はなく，また塞栓材併用なしで実施した臨床試験と比べて，臨床上問題となる新たな有害事象や肝臓に対して大きな問題となる有害事象も認められなかった。

一般的にTACE後に半数以上に発現することが知られている副作用として，投与直後の一過性の悪心・嘔吐，投与部位疼痛，発熱などがあり，また，TACE後にAST，ALTなどが一過性に上昇することが知られている[10]。塞栓材を併用せずに実施した本剤の臨床試験でもこれらの有害事象は発現しており，本治験でも半数以上の患者で発現した。

TACEで塞栓材を併用した場合，その血流遮断効果から併用しない場合に比べ抗腫瘍効果は高まるが，その一方で併用しない場合に比べ肝臓への影響が強く現れることが懸念される[8,11]。本治験では，肝動脈内投与による肝臓への影響として，ALT増加，AST増加，血中アルブミン減少，血中ビリルビン増加などの一過性の有害事象が発現したが，その程度および種類は塞栓材を併用せずに行った臨床試験と比較して大きく異なるものではなかった。

その他に，cisplatinで懸念されている腎障害，骨髄抑制などに関連する臨床検査値について特記すべき変動は認められなかった。これら全身性の有害事象が軽度であることは，塞栓材を併用せずに実施した臨床試験と同様であった。なお，塞栓材を併用せずに実施した臨床試験では好酸球増多が高頻度に認められているが，本治験でも同様に認められた。

結　語

SM-11355に塞栓材を併用した場合の安全性および有効性を肝細胞癌患者10名で検討した。塞栓材の併用により抗腫瘍効果は増強される可能性が示唆され，安全性では肝細胞癌の治療上問題となるような有害事象は認められなかった。

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CLINICAL STUDIES

HBcrAg is a predictor of post-treatment recurrence of hepatocellular carcinoma during antiviral therapy

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Keywords
covalently closed circular DNA – HBcrAg – HCC recurrence – nucleot(s)ide analogue – portal vein invasion

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Abstract

Background/Aims: The recurrence rate of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) is high even in patients receiving curative therapy. In this study, we analysed the risk factors for tumour recurrence after curative therapy for HBV-related HCC while under treatment with nucleot(s)ide analogues (NAs) by measuring serum HBcrAg and intrahepatic covalently closed circular DNA (cccDNA) levels to elucidate the viral status associated with HCC recurrence. *Methods:* We enrolled 55 patients who developed HCC during NA therapy and underwent either curative resection or percutaneous ablation for HCC. *Results:* Hepatocellular carcinoma recurred in 21 (38%) of the patients over a period of 2.2 (range, 0.2–7.4) years. In multivariate analysis, serum HBcrAg levels $\geq$4.8log U/ml at the time of HCC diagnosis (hazard ratio, 8.96; 95% confidential interval, 1.94–41.4) and portal vein invasion (3.94, 1.25–12.4) were independent factors for HCC recurrence. The recurrence-free survival rates of the high cccDNA group were significantly lower than those of the low cccDNA group only in patients who underwent resection ($P=0.0438$). A positive correlation ($P=0.028$; $r=0.479$) was observed between the intrahepatic cccDNA and the serum HBcrAg levels at the incidence of HCC. *Conclusion:* HBcrAg is a predictor of the post-treatment recurrence of HCC during antiviral therapy. Serum HBcrAg and intrahepatic cccDNA suppression by NAs may be important to prevent HCC recurrence.

Worldwide, an estimated 400 million people are infected with hepatitis B virus (HBV) persistently, and one million people die of decompensated cirrhosis and/or hepatocellular carcinoma (HCC) annually (1, 2). Recently, oral nucleot(s)ide analogues (NAs) have been used as the mainstay therapeutic strategy against chronic hepatitis B. Five such antiviral agents have been approved, and range in the profundity and rapidity of HBV DNA suppression, barrier to resistance and side-effect profile (3–10). Lamivudine (LAM) was the first NA to be approved for treating chronic hepatitis B, followed by adefovir dipivoxil (ADV) and entecavir (ETV), in Japan. However, a major problem with long-term LAM treatment is the potential development of drug resistance, mainly caused by mutation of the thyrosine–methionine–aspartic acid–aspartic acid (YMDD) motif of reverse transcriptase (11, 12). For preventing breakthrough hepatitis induced by LAM-resistant mutants, additional ADV administration has been recommended (13, 14).

The methods for monitoring the treatment response include measurements of the serum alanine transaminase (ALT) levels, HBV DNA levels, HBeAg and antibody levels, HBsAg and antibody levels and liver histology. Other serum markers have been reported to be useful for monitoring the effect of antiviral therapy (15, 16). Recently, a new assay was developed for detecting the HBcrAg, consisting of HBcAg, HBeAg and a 22 kDa precore protein coded with the precore/core gene (17, 18). Because NAs have no inhibiting action on the transcription and translation activities of viral mRNA, HBcAg- and HBeAg-related proteins continue to be produced for a certain period of time in spite of the achievement of adequate suppression of the viral DNA synthesis. Therefore, HBcrAg is a viral marker independent of HBV DNA for monitoring the antiviral effect of NAs (19). In addition, recent reports have indicated another interesting aspect of serum HBcrAg levels: these levels were found to be correlated with intrahepatic covalently closed circular DNA (cccDNA) levels and could be a surrogate marker of the intrahepatic cccDNA pool (20, 21). This phenomenon may be explained by the fact that the production of HBcrAg depends on the

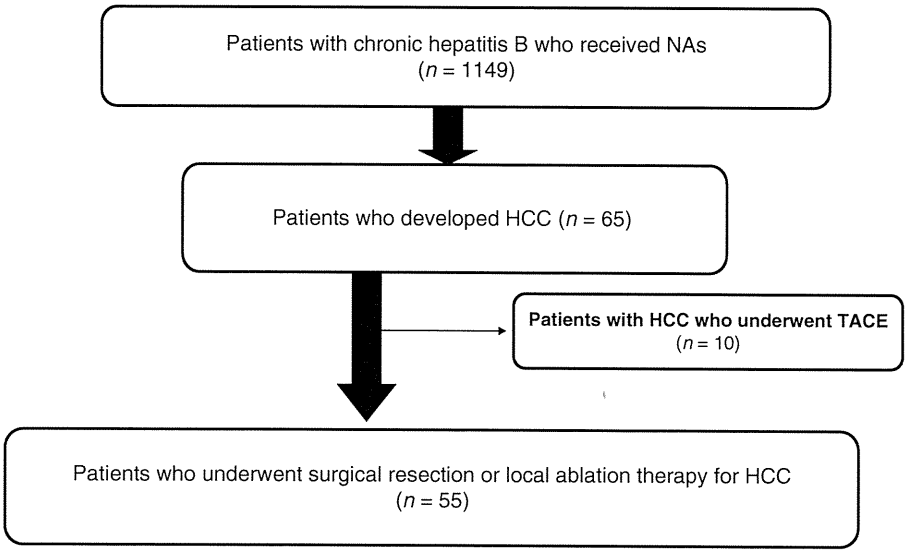

Fig. 1. The study protocol. HCC, hepatocellular carcinoma; NAs, nucleot(s)ide analogues; TACE, transcatheter arterial chemoembolization.

transcription of mRNA from cccDNA, and that cccDNA still remains in high levels during treatment with NAs.

Although patients with HBV-related cirrhosis have a significantly high risk of developing HCC, NA therapy can delay the progression of liver disease and reduce the risk of HCC in patients with cirrhosis by strong viral suppression (22, 23). Nevertheless, a few cases develop HCC during NA therapy at a constant rate (3–12%) (22, 24–26). The recurrence rate of HBV-related HCC after curative resection is estimated to be high, and is associated with viral factors, including HBeAg positivity and the viral load before surgery, besides host and tumour factors, but these findings were demonstrated in the absence of antiviral therapy (27–30). However, almost all patients, receiving NAs, showed negativity of serum HBV DNA. And so, we made the hypothesis that intrahepatic viral status, such as intrahepatic cccDNA and serum HBcrAg levels of its surrogate maker, might have an impact on tumour recurrence during NA therapy.

In this study, we examined the risk factors for tumour recurrence after curative resection and ablation for HBV-related HCC during NA therapy by measuring the serum HBcrAg and intrahepatic cccDNA levels with the aim to elucidate the viral status, persistent despite suppressive therapy, associated with HCC recurrence, in addition to the host and tumour factors reported in the past.

Patients and methods

Patients

Over a period of 13 years, from September 1995 to September 2008, 1149 patients with chronic hepatitis B received NA therapy, including LAM, ADV and ETV, at the Department of Hepatology, Toranomon Hospital, Metropolitan Tokyo. Of the 1149 patients, 65 developed HCC after the start of NA therapy from February 2001 to June 2009. Of the 65 consecutive patients, 55 underwent radical therapy, including either resection or percutaneous ablation as the initial therapy for HCC. These 55 patients were enrolled in this cohort study (Fig. 1). The median duration from the start of NA therapy to the development of HCC was 2.2 (range, 0.2–7.4) years. The exclusion criteria were (i) patients co-infected with hepatitis C, delta or human immunodeficiency virus and (ii) a history of other liver diseases such as autoimmune hepatitis, alcoholic liver disease or metabolic liver disease.

The diagnosis of HCC was predominantly based on imaging, including dynamic computed tomography, magnetic resonance imaging and/or digital subtraction angiography. When the hepatic nodule did not show the typical imaging features, fine needle aspiration biopsy was performed, followed by histological examination and diagnosis. The physicians and surgeons usually discussed the preferred choice of treatment for each patient. Hepatic resection was mainly performed for patients categorized as Child–Pugh grade A or B liver function, and had no serious complications. Percutaneous ablation was performed for patients with surgical contraindications or for those who did not prefer to undergo hepatic resection by using two different devices: the cool-tip system (Tyco Healthcare Group LP, Burlington, VT, USA) and the radiofrequency tumour coagulation system (RTC system; Boston-Scientific Japan Co., Tokyo, Japan). The term curative treatment was used to indicate that no tumours were left in the remnant liver, irrespective of the width of the margin around the tumour, confirmed using intra-operative ultrasonography, combined ultrasonography and dynamic computed tomography 1 month after the resection or ablation. Serum samples were collected from all patients before and after

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the treatment for HCC and stored in −80 °C. Liver tissue from patients who underwent resection was collected, rapidly frozen and stored in −80 °C. Written informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval by the institution's human research committee.

Antiviral therapy

Forty-seven patients received 100 mg LAM daily, and drug-resistant YMDD mutants developed in 26 (55%) of these patients, accompanied by an increase in HBV DNA ≥1log copies/ml. Seventeen of the 26 patients received 10 mg ADV in addition to LAM (100 mg) daily. The remaining nine continued to receive LAM monotherapy because of the lack of approval for ADV administration in Japan at the time, but received ADV with LAM after approval was obtained during the HCC posttreatment period. Eight NA-naïve patients received 0.5 mg ETV daily. These antiviral therapies were continued after the resection or percutaneous ablation.

Follow-up and HCC recurrence

The patients were followed for liver function and virological markers of HBV infection monthly, as well as blood counts and tumour makers including α-fetoprotein and des-γ-carboxylprothrombin. They also underwent ultrasonography or helical dynamic computed tomography every 3 months. Cirrhosis was diagnosed by laparoscopy or liver biopsy or by the clinical data, imaging modalities and portal hypertension. The median observation period after HCC treatment for the entire cohort was 2.7 years (range, 0.3–8.4 years). HCC recurrence was diagnosed by the typical hypervascular characteristics on angiography and/or histological examination with fine needle biopsy specimens, in addition to certain features on computed tomography and ultrasonography.

Markers of HBV infection

HBeAg was determined by enzyme-linked immunosorbent assay using a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). HBV DNA was quantitated using the Amplicor monitor assay (Roche Diagnostics, Tokyo, Japan) with a dynamic range over 2.6–7.6log copies/ml or COBAS TaqMan HBV v.2.0 (Roche Diagnostics) with a dynamic range over 2.1–9.0log copies/ml. Serum HBV DNA levels were measured using the Amplicor assay at both the start of NA therapy and the diagnosis of HCC and using the TaqMan assay at the diagnosis of HCC. For statistical analysis, the value of that HBV DNA was tentatively set at 2.1 if HBV DNA levels were under 2.1log copies/ml. HBV genotypes were determined serologically by the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the seven major genotypes (A–G), using a commercial kit (HBV Genotype EIA; Institute of Immunology). YMDD mutants were determined by polymerase chain reaction-based enzyme-linked minisequence assay using a commercial kit (Genome Science Laboratories, Tokyo, Japan).

HBcrAg measurement

Serum HBcrAg levels were measured using a CLEIA HBcrAg assay kit (Fujirebio Inc., Tokyo, Japan) with a fully automated analyser system (Lumipulse System; Fujirebio Inc.) as described previously (21). In brief, 150 µl of serum was incubated with 150 µl of pretreatment solution containing 15% sodium dodecyl sulphate at 60 °C for 30 min. After heat treatment, 120 µl of pretreated specimen was added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with monoclonal antibody mixture (HB44, HB61 and HB114) against denatured HBcAg, HBeAg and the 22 kDa precore protein. After 10 min of incubation at 37 °C and washing, further incubation was carried out for 10 min at 37 °C with alkaline phosphatase conjugated with two kinds of monoclonal antibodies (HB91 and HB110) against denatured HBcAg, HBeAg and the 22 kDa precore protein. After washing, 200 µl of substrate solution [3-(2′-spiroada-mantan)-4-methoxy-4-(3′-phosphoryloxy)pheny1-1,2-dioxetane disodium salt] (Applied Biosystems, Bedford, MA, USA) was added to the test cartridge, which was then incubated for 5 min at 37 °C. The relative chemiluminescence intensity was measured, and the HBcrAg concentration was calculated by a standard curve generated using a recombinant pro-HBeAg (amino acids −10 to 183 of the precore/core gene product). The HBcrAg concentration was expressed in U/ml, which is defined as the immunoreactivity of 10 fg/ml of recombinant pro-HBeAg. In this study, the HBcrAg values were expressed as log U/ml, and the cut-off value was set at 3.0 log U/ml. For the statistical analyses, HBcrAg-negative cases were calculated as 3.0log U/ml.

Intrahepatic cccDNA measurement

Intrahepatic cccDNA levels were analysed as described previously (21). In brief, liver specimens surrounding the tumour tissue were obtained and stored at −80 °C before DNA extraction. HBV DNA was extracted using a QIAamp DNA Mini Kit (Qiagen KK, Tokyo, Japan). The concentration of purified DNA was based on the absorbance at 260 nm. For this study, two oligonucleotide primers cccF2 (5′-cgtctgtgccttctcatctga-3′, nucleotides 1424–1444) and cccR4 (5′-gcacagcttggaggcttgaa-3′, nucleotides 1755–1737) and probe cccP2 (5′-VIC-accaatttat gcctacag-MGB-3′, nucleotides 1672–1655) were designed using PRIMER EXPRESS software (Applied Biosystems, Foster City, CA, USA) to flank the direct repeat region between the hepatitis B core and the polymerase gene. The use of cccF2 and cccR4, oligonucleotide primers spanning the direct repeat region of the HBV genome, allows the polymerase chain reaction of native viral DNA in the

Dane particle to block the amplification of products, because the partially double-stranded HBV DNA is disrupted in the direct repeat region. Twenty-five microlitres of extracted DNA (0.5 μg) was detected with the sequence detector system (ABI 7900HT; Applied Biosystems) in 50 μl of a PCR mixture containing TaqMan universal PCR Master Mix (Applied Biosystems), 300 nmol of each primer and 250 nmol of the probe. After initial activation of uracil-*N*-glycosylase at 50 °C for 2 min, AmpliTaq Gold (Applied Biosystems) was activated at 95 °C for 10 min. The subsequent PCR conditions consisted of 45 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 90 s per cycle (SRL Inc., Tokyo, Japan).

Statistical analyses

Standard statistical measures and procedures were used. Correlations between two variables were tested using Pearson's correlation analysis. Cox regression analysis was used to assess significant associations of the risk factors with tumour recurrence after HCC treatment. All factors found to be at least associated with recurrence ($P < 0.05$) were tested by multivariate analysis. Independent factors, associated with HCC recurrence, were calculated using stepwise Cox regression analysis. The cumulative recurrence-free survival rates after HCC treatment were analysed using the Kaplan–Meier method, and differences in the curves were tested using the log-rank test. A P value of < 0.05 in a two-tailed test was considered significant. Data analysis was performed with SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics at the start of NA therapy and HCC incidence

Table 1 presents a comparison of the patient characteristics at the start of NA therapy and the time of HCC diagnosis. Almost all the patients (93%) enrolled in this study had HBV genotype C. One patient had genotype B, and the genotypes of three patients could not be determined. The rate of HBV DNA disappearance from serum in all the patients was 64% (35/55; Amplicor monitor assay, < 2.6log copies/ml) and 51% (28/55; TaqMan assay, < 2.1log copies/ml), that of aspartate aminotransferase (AST) normalization (< 32 IU/L) was 56% (31/55) and that of ALT normalization (< 42 IU/L) was 71% (39/55) at the incidence of HCC. YMDD mutants were detected in 30 of 47 patients at the beginning of LAM monotherapy, and virological breakthrough (VBT), accompanied by an increase in HBV DNA (≥ 1log copies/ml), occurred in 26 patients with YMDD mutants by the diagnosis of HCC. Seventeen of these patients received ADV with LAM. No resistant mutation to ADV (rtA181T/S, rtN236T) occurred in patients receiving the combination therapy. Further, no drug-resistant mutant

Table 1. Patient characteristics at the start of nucleot(s)ide analogue therapy and the incidence of hepatocellular carcinoma

Characteristics	Start of NA therapy	Time of HCC Dx
Age (years)	51 (32–73)	54 (35–75)
Gender (male:female)	45:10	45:10
AST level (IU/L)	69 (27–195)	31 (16–207)
ALT level (IU/L)	78 (23–368)	29 (10–267)
Platelet count (10^5/mm^3)	11.4 (3.1–31.3)	12.9 (3.6–30.1)
Serum albumin level (g/dl)		3.8 (3.1–4.4)
Serum bilirubin level (mg/dl)		0.9 (0.4–2.4)
Prothrombin time (%)		90.8 (59–112)
Indocyanine green retention rate at 15 min (%)		14.5 (4–53)
Child–Pugh (A:B)		49:6
HBV genotype		
C	51 (93%)	51 (93%)
Others	4	4
HBeAg (+)	29 (53%)	23 (42%)
HBV DNA (log copies/ml)	7.1 (< 2.6 to > 7.6)	< 2.1 (< 2.1 to 8.5)
HBcrAg level (log U/ml)	6.6 (3.3 to > 6.8)	5.0 (< 3.0 to > 6.8)
Antiviral agents (LAM:LAM+ADV:ETV)	47:0:8	30:17:8
Duration of NA therapy before the incidence of HCC (years)		2.2 (0.2–7.4)
α-fetoprotein level (ng/dl)	6 (2–263)	4 (1–282)
Des-γ-carboxylprothrombin level (mAU/ml)		22 (< 10–933)
Tumour diameter (mm)		22 (7–60)
Tumour number (solitary:multiple)		50:5
Portal vein invasion (positive:negative)		49:6
TNM stage (I:II:III:IV)		25: 24: 5: 1
HCC treatment (resection:ablation)		37:18

Values are expressed as the median and range (parenthetically) or the number and percentage (parenthetically).
ADV, adefovir dipivoxil; ETV, entecavir; HBV DNA, hepatitis B virus DNA; HCC, hepatocellular carcinoma; LAM, lamivudine; NA, nucleot(s)ide analogues.

was detected in the NA-naïve patients receiving ETV monotherapy.

Correlation between serum HBcrAg and serum HBV DNA levels at the incidence of HCC

The median serum HBcrAg value was 6.6log U/ml (range, 3.3 to > 6.8) at the start of NA therapy and 5.0log U/ml (range, < 3.0 to > 6.8) at the time of HCC diagnosis. We observed a positive correlation ($P < 0.001$; $r = 0.610$) between the levels of HBcrAg and HBV DNA in serum at the time of HCC diagnosis (Fig. 2A).

HBcrAg was detectable in 23 (82%) of 28 patients with undetectable HBV DNA levels using TaqMan assay and was > 4.8log U/ml in eight (29%) of 28 patients. In contrast, serum HBV DNA was detectable in spite of undetected HBcrAg in only two patients. Then, we examined the correlation between the serum HBcrAg levels at the time of HCC diagnosis and the antiviral effect. The median duration of on-treatment undetected serum HBV DNA was 1.1 years (range, 0.1–4.8) before the first diagnosis of HCC. As shown in Figure 2B, we observed a significant negative correlation between the levels of HBcrAg in serum at the time of HCC diagnosis and the duration of undetected HBV DNA in serum just before the first diagnosis of HCC ($P < 0.001$; $r = -0.568$).

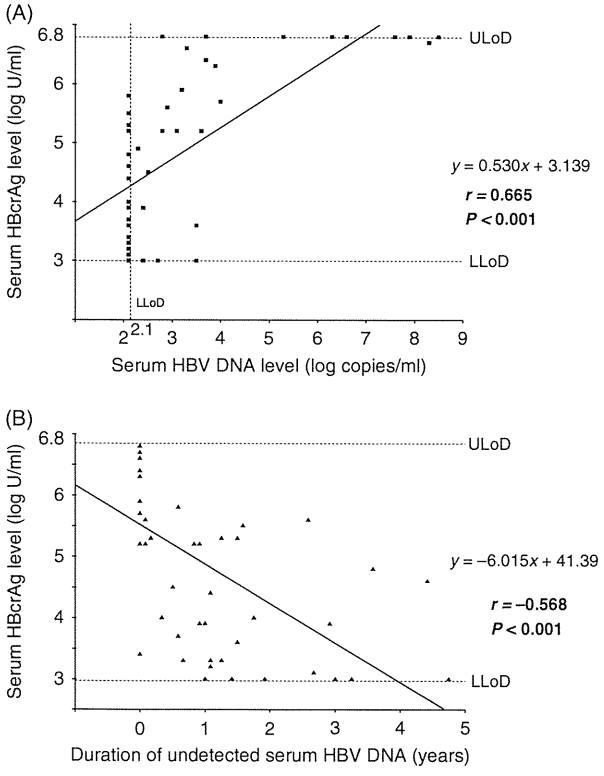

Fig. 2. (A) Correlation between serum HBcrAg and hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient. (B) Correlation between serum HBcrAg levels at the time of HCC diagnosis and the duration of undetected serum HBV DNA (< 2.6log copies/ml).

Factors associated with HCC recurrence

Hepatocellular carcinoma recurred in 21 (38%) of the 55 patients, 17 (46%) of 37 patients who had undergone resection and four (22%) of 18 patients who had undergone ablation. Because a proportion of patients who had undergone resection with TNM Stage II or over (24 of 37 patients) was greater than ablation (six of 18), there were more patients who had HCC recurrence after resection than ablation. Eight factors were associated with the recurrence in univariate analysis: HBeAg positivity at the start of NA therapy, HBV DNA ≥2.1log copies/ml, HBcrAg level ≥4.8log U/ml, AST level ≥50 IU/L, ALT level ≥40 IU/L, tumour multiplicity, portal vein invasion at the time of HCC diagnosis and HCC treatment. In the multivariate analysis, HBcrAg level ≥4.8log U/ml and portal vein invasion were independent risk factors for the recurrence of HCC (Table 2). The cumulative recurrence-free survival rates in patients with ≥4.8log U/ml HBcrAg levels at the time of HCC diagnosis were 70% at 1 year, 35% at 3 years and 28% at 5 years. In contrast, the rates in patients with < 4.8log U/ml HBcrAg levels were 96% at 1 year, 89% at 3 years and 89% at 5 years. The recurrence-free survival rates of the high HBcrAg group (≥4.8log U/ml) were significantly lower than those of the low HBcrAg group (< 4.8log U/ml; $P < 0.001$), as shown in Figure 3A. Then, the cumulative recurrence-free survival rates in patients with ≥2.1log copies/ml HBV DNA levels at the time of HCC diagnosis were 70% at 1 year, 44% at 3 years and 39% at 5 years. In contrast, the rates in patients with < 2.1log copies/ml HBV DNA levels were 93% at 1 year, 76% at 3 years and 76% at 5 years. The recurrence-free survival rates of the positive HBV DNA group (≥2.1log copies/ml) were significantly lower than those of the negative HBV DNA group (< 2.1log copies/ml; $P = 0.007$), as shown in Figure 3B. The cumulative recurrence-free survival rates were 33% at 1 year and 33% at 2 years with portal vein invasion, and 87% at 1 year, 73% at 2 years and 64% at 3 years without invasion. Three of the six patients with portal vein invasion died of recurrent HCC.

Correlation between intrahepatic cccDNA and serum HBV DNA levels at the incidence of HCC

We measured intrahepatic cccDNA using liver specimens from 22 of 37 patients who underwent resection. The median intrahepatic cccDNA value was 4.2log copies/μg (range, 3.0–5.0). As shown in Figure 4A and B, we observed significant positive correlations between the levels of intrahepatic cccDNA and HBV DNA in serum ($P = 0.019$; $r = 0.486$) and between the levels of intrahepatic cccDNA and HBcrAg in serum at the time of HCC diagnosis ($P = 0.028$; $r = 0.479$). Twenty-eight patients who underwent resection had early- or intermediate-stage

Table 2. Risk factors for hepatocellular carcinoma recurrence

	Univariate analysis		Multivariate analysis	
Factors	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
Start of NA therapy				
Age (≥50 years)	1.79 (0.65–4.91)	0.257		
Gender (female)	0.98 (0.32–2.97)	0.981		
HBeAg(+)	2.85 (1.03–7.88)	**0.044**		
HBV DNA (≥6.0log copies/ml)	1.75 (0.50–6.07)	0.378		
AST level (≥50 IU/L)	1.09 (0.42–2.85)	0.862		
ALT level (≥70 IU/L)	1.09 (0.42–2.85)	0.862		
Platelet count (< 1.2×10^5 cells/mm^3)	2.56 (0.96–6.85)	0.061		
α-fetoprotein level (≥100 ng/ml)	0.99 (0.13–7.66)	0.996		
Time of HCC diagnosis				
Duration of NA therapy (≥2 years)	1.19 (0.49–2.88)	0.698		
HBeAg(+)	1.53 (0.63–3.70)	0.343		
HBV DNA (≥2.1log copies/ml)	3.36 (1.32–8.55)	**0.011**		
HBcrAg level (≥4.8log U/ml)	10.6 (2.45–46.1)	**0.002**	**8.96 (1.94–41.4)**	**0.005**
YMDD mutants (present:absent)	0.84 (0.35–2.03)	0.838		
AST level (≥50 IU/L)	2.44 (1.01–5.89)	**0.047**		
ALT level (≥40 IU/L)	2.44 (1.01–5.87)	**0.047**		
Platelet count (< 10^5 cells/mm^3)	2.20 (0.81–6.02)	0.123		
Serum albumin level (< 3.5 g/dl)	1.39 (0.53–3.63)	0.505		
Serum bilirubin level (≥1.5 mg/dl)	1.11 (0.62–2.00)	0.713		
Prothrombin time (< 80%)	2.23 (0.51–9.82)	0.286		
Child–Pugh (B)	0.70 (0.16–3.04)	0.634		
Indocyanine green retention rate at 15 min (≥30%)	0.58 (0.17–1.99)	0.389		
α-fetoprotein level (≥100 ng/ml)	1.81 (0.74–4.44)	0.194		
Des-γ-carboxylprothrombin level (≥100 mAU/ml)	2.09 (0.81–5.39)	0.129		
Tumour size (≥21 mm)	2.02 (0.81–5.07)	0.133		
Tumour number (multiple)	3.94 (1.29–12.1)	**0.016**		
Portal vein invasion	5.39 (1.69–17.2)	**0.004**	**3.94 (1.25–12.4)**	**0.019**
TNM stage (≥II)	2.08 (0.85–5.10)	0.110		
HCC treatment (resection)	3.10 (1.05–9.09)	**0.041**		

The bolded numbers: statically significant.

ALT, alanine transaminase; AST, aspartate aminotransferase; CI, confidence interval; HBV DNA, hepatitis B virus DNA; NA, nucleot(s)ide analogues; YMDD, thyrosine–methionine–aspartic acid–aspartic acid.

HCC (tumour diameter < 50 mm, absence of vascular invasion and well/moderately differentiated). In 17 of these patients, the intrahepatic cccDNA levels were measured using the resected specimens. The recurrence-free survival rates of the high cccDNA group (≥4.3log copies/μg) were significantly lower than those of the low cccDNA group (< 4.3log copies/μg; $P = 0.0438$), as shown in Figure 4C.

Comparison of the serum HBcrAg levels and the patient characteristics

We examined whether the serum HBcrAg levels at the time of HCC diagnosis were correlated with the baseline parameters before antiviral therapy. The HBcrAg levels were compared with the baseline HBeAg-positive and HBeAg-negative status and with the baseline HBV DNA levels ≥6.0log and < 6.0log copies/ml (Fig. 5). The HBcrAg levels were significantly higher in patients who were positive for HBeAg (median value: 5.6 vs. 3.6log U/ml; $P = 0.001$) and the baseline HBV DNA levels ≥6.0log copies/ml (median value: 5.2 vs. 3.3log U/ml; $P = 0.012$). There was no correlation between the other baseline parameters at the start of NA therapy and the serum HBcrAg levels at the time of HCC diagnosis. Then, we examined whether the serum HBcrAg levels at the time of HCC diagnosis were associated with on-treatment drug resistance during antiviral therapy. Figure 6 shows the comparison of the serum HBcrAg levels at the time of HCC diagnosis with or without the emergence of YMDD mutants and VBT before the development of HCC. The HBcrAg levels were marginally higher in patients with emergent YMDD mutants (median value: 5.2 vs. 3.8log U/ml; $P = 0.051$) and significantly higher in those with VBT (median value: 5.2 vs. 3.9log U/ml; $P = 0.006$). There was no correlation between serum HBcrAg at the time of HCC diagnosis and age of patients or tumour factors.

Discussion

In this study, we examined whether the intrahepatic cccDNA and HBcrAg levels as substitutes for cccDNA are associated with HCC recurrence in patients who

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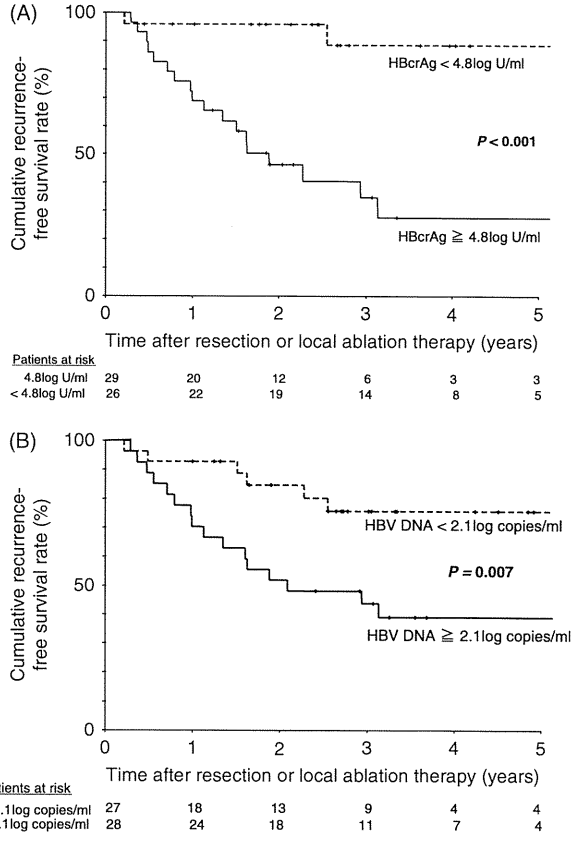

Fig. 3. (A) Kaplan–Meier life table for the cumulative recurrence-free survival rates by the serum HBcrAg levels and comparison by the log-rank test. (B) Kaplan–Meier life table for the cumulative recurrence-free survival rates by the serum hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient and comparison by the log-rank test.

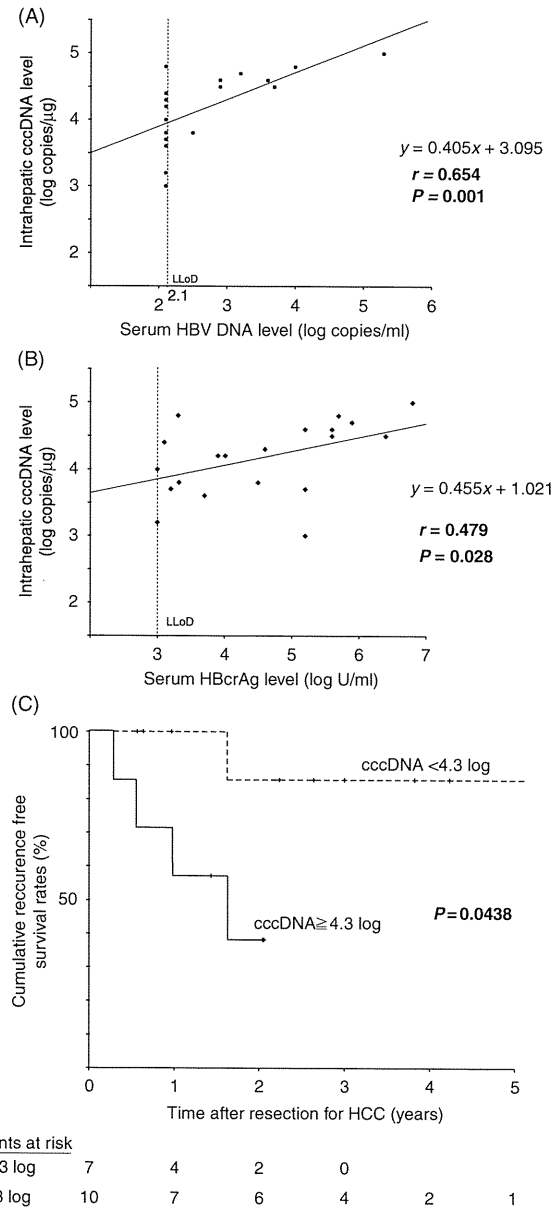

Fig. 4. (A) Correlation between intrahepatic covalently closed circular DNA (cccDNA) and serum hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient who underwent resection ($n = 22$). (B) Correlation between intrahepatic cccDNA and serum HBcrAg levels at the time of HCC diagnosis. (C) Kaplan–Meier life table for the cumulative recurrence-free survival rates by the intrahepatic cccDNA levels in patients with early- or intermediate-stage HCC ($n = 17$).

developed HCC after the commencement of NA therapy and underwent radical therapy for HCC. The recurrence rates of HCC were high in patients with high levels of intrahepatic cccDNA and serum HBcrAg. In particular, HBcrAg levels were measurable by using serum samples and clinically useful.

Nucleot(s)ide analogues, including LAM, ADV and ETV, are widely used for the treatment of chronic hepatitis B, and reportedly reduce the development of HCC in such patients (22, 23). Although few events of HCC development occur during NA therapy (24–26), analysis of a large number of patients is needed to examine the risk factors for HCC. We could clarify the risk factors associated with the development of primary HCC after radical therapy by enrolling patients who underwent radical therapy for HCC in spite of their small number. High HBV loads in serum have been reported to be associated with HCC recurrence after resection or radical therapy in NA-naïve patients (27–31), but no study has demonstrated the viral risk factors of recurrence in patients receiving NAs. The novel finding of this study is that serum HBcrAg and intrahepatic cccDNA levels are predictors of HCC recurrence in patients radically treated for HCC during NA therapy.

In this study, the serum HBV DNA levels at the time of HCC diagnosis were associated with recurrence by univariate analysis. However, the serum HBcrAg level was the only viral factor associated with recurrence in multivariate analysis. There are two possible reasons for the

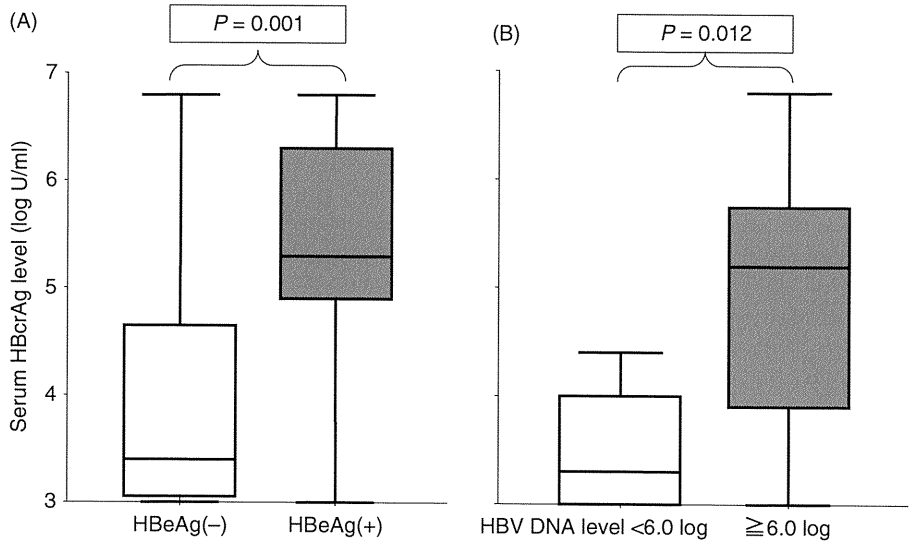

Fig. 5. Comparison of serum HBcrAg levels at the time of hepatocellular carcinoma diagnosis by the characteristics at the start of nucleot(s)ide analogue therapy (A) in patients with or without HBeAg and (B) in those with hepatitis B virus DNA (HBV DNA) levels < 6.0log or ≥ 6.0log copies/ml.

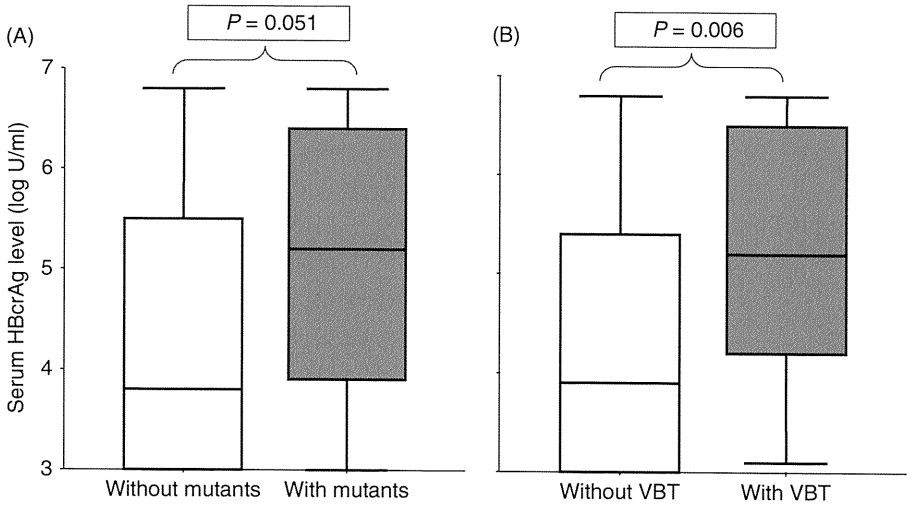

Fig. 6. Comparison of serum HBcrAg levels at the time of hepatocellular carcinoma (HCC) diagnosis (A) with or without thyrosine–methionine–aspartic acid–aspartic acid mutants and (B) virological breakthrough (VBT) before the development of HCC.

different results between past studies and the current study. Although serum HBV DNA was undetectable using TaqMan assay at the time of HCC diagnosis in 51% of the patients, who received NAs, serum HBcrAg was undetectable in only 18% of these patients. The other reason is that it was easy to identify the viral risk factors (e.g. HBeAg positivity) by measuring the serum HBcrAg level because the detection of HBcrAg enables the detection of HBcAg, HBeAg and the 22 kDa precore protein coded with the precore/core gene. The high recurrence rate of HCC after curative resection and ablation is attributable to two principal characteristics: intrahepatic metastasis and *de novo* multicentric carcinogenesis (32). It is assumed that a high viral load increases the risk of multicentric recurrence in the liver remnant in patients without optimal viral suppression by NA therapy. Recently, it was reported that the HBV load is associated with late recurrence over 2 years (30). On examining our cohort as per the recent report, high HBcrAg levels were found to be associated with late recurrence (data not shown). Consequently, we consider that HBcrAg is a more useful marker of HBV-related HCC recurrence than HBV DNA during NA therapy.

Nucleot(s)ide analogues are potent inhibitors of HBV replication, and can induce a rapid and drastic reduction in peripheral HBV DNA, seroclearance of HBeAg and remission of hepatic inflammation. Because of the stability of cccDNA in infected cells, the decline of

intrahepatic cccDNA levels is slower than that of serum HBV DNA levels during NA administration (15, 16). We found that suppression of cccDNA by NAs could prevent the development of recurrent primary HCC. Because cccDNA provides the template for pregenomic and viral messenger RNA-encoded viral proteins (33–35), the transcriptional activity of cccDNA may induce carcinogenesis. Further research is required to validate this hypothesis. Serum HBcrAg can be a surrogate marker of the intrahepatic cccDNA pool because of the viral proteins transcribed through messenger RNA from cccDNA (20, 21). Therefore, we consider that serum HBcrAg reflects the intrahepatic viral status more accurately than serum HBV DNA. Recently, Chan *et al.* (36) showed that serum HBsAg quantification could reflect intrahepatic cccDNA in patients treated with peginterferon and LAM combination therapy. They also indicated that reduction in HBsAg had good correlation with reduction in cccDNA. We tried to measure HBsAg levels at the start of NA therapy and the time of HCC diagnosis using a commercial assay (chemiluminescent immunoassay). However, HBsAg levels declined very slowly during NAs monotherapy in this study (data not shown). Brunetto *et al.* (37) showed that mean reduction for 48 weeks in HBsAg was 0.02log IU/ml in patients treated with LAM monotherapy, different from peginterferon therapy. Meanwhile, the median reduction from the start of NA to the diagnosis of HCC in HBcrAg was 1.4logU/ml in this study (Table 1). It seems that HBcrAg is a superior on-treatment risk predictor (e.g. tumour recurrence) to HBsAg during NAs monotherapy in terms of reduction of titres in each assay. HBcrAg is also more useful in terms of needless to serum sample dilution. As HBcrAg levels can be measured from serum samples, they are clinically useful, compared with the measurement of cccDNA, which requires liver specimens. It is not practical to carry out liver biopsy and the measurement of cccDNA for patients who have normal AST/ALT levels and viral suppression during antiviral therapy. Liver specimens cannot be also taken from patients who undergo ablation therapy for HCC. The measurement of serum HBcrAg levels in these patients is helpful to indirectly estimate the status of intrahepatic cccDNA. In the future, it is necessary to investigate whether HBcrAg in patients receiving NAs can be a predictor of primary carcinogenesis.

Previous studies have indicated that the rates of intrahepatic cccDNA loss and serum HBcrAg loss differ from serum HBV DNA loss under NA therapy, with the former two being much slower (15, 16, 19). In this study, the period of serum HBV DNA loss was longer, with lower intrahepatic cccDNA and serum HBcrAg levels (Fig. 2B). Therefore, these findings suggest that a long period of time is required to prevent the development of recurrent primary HCC by viral suppression under antiviral therapy. In contrast, the serum HBcrAg levels at the time of HCC diagnosis were higher in patients with emergent LAM-resistant mutants and subsequent VBT than in patients without mutants and VBT (Fig. 6). This result suggests that it is important to administer a potent NA early for drug-resistant strains and suppress viral replication to prevent subsequent carcinogenesis. Although we evaluated the relationship between the development of primary HCC and serum HBcrAg levels by a case–control study, the serum HBcrAg levels at the commencement of NA therapy and 1 year later were not associated with the development of primary HCC (unpublished data). This finding is attributable to the slow decline of the serum HBcrAg levels during antiviral therapy. The measurement of HBcrAg at intervals of 3–6 months may be helpful to predict the development of HCC. However, further studies are needed to confirm the finding.

In summary, HBcrAg is a predictor of the post-treatment recurrence of HCC during antiviral therapy. Measurement of the serum HBcrAg level is simple and useful because it reflects the intrahepatic viral status. Further, intrahepatic cccDNA and serum HBcrAg suppression by NAs is important to prevent HCC recurrence.

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Original Article

Administration of interferon for two or more years decreases early stage hepatocellular carcinoma recurrence rate after radical ablation: A retrospective study of hepatitis C virus-related liver cancer

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Background: Since hepatocellular carcinoma often recurs after surgical resection or radiofrequency ablation, we analyzed a retrospective large cohort of patients with small hepatocellular carcinoma caused by hepatitis C virus (HCV).

Methods: Among 379 patients with HCV RNA-positive small hepatocellular carcinoma (multiple up to three nodules, 3 cm or less each), 77 received interferon-alpha injection and 302 received no anti-viral therapy.

Results: Four patients (5.2%) attained sustained virological response (SVR). Cumulative recurrence rates in the treated and untreated groups were 41.1% and 57.5% at the end of the third year, and 63.0% and 74.5% at the fifth year, respectively ($P = 0.013$). Fifth year-recurrence rates in treated group were 25.0% in SVR, 85.7% in biochemical response, 71.1% in no response, and 46.7% in patients with continuous administration. When four patients with SVR were excluded, recurrence rates in short-term interferon therapy (<2 years) and long-term therapy (≥2 years) were 46.2% and 39.3% at the third year, and 66.2% and 57.4% at the fifth year, respectively ($P = 0.012$). Multivariate analysis showed that long-term interferon therapy significantly decreased recurrence rate (hazard ratio for interferon <2 years 0.80, interferon ≥2 years 0.60, $P = 0.044$), after adjustment with background covariates including indocyanine green retention rate ($P = 0.018$), alpha-fetoprotein ($P = 0.051$), and tumor treatment ($P = 0.066$).

Conclusion: A long-term administration of low-dose interferon significantly decreased recurrence of hepatocellular carcinoma after surgical resection or radiofrequency ablation.

Key words: hepatitis C, hepatocellular carcinoma, Interferon, prevention, recurrence

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) remains one of the most common cancers, and cause of cancer death, worldwide. Since the recurrence rate of HCC is high even after potentially curative therapies with surgical resection or radiofrequency ablation (RFA) therapy, suppression of recurrence is of great importance for prolonging the life of patients with hepatitis C virus (HCV)-related liver disease. This high recurrence rate, after curative therapy, was explained by occult intra-hepatic metastasis of HCC or by multi-centric carcinogenesis in the setting of chronic viral hepatitis or liver cirrhosis.[1,2]

Interferon (IFN) is effective in reducing hepatocellular carcinogenesis rate through suppression of necro-inflammatory process and in eliminating HCV in some patients with chronic hepatitis C and cirrhosis. Although IFN proves to be valuable in suppression of the risk of carcinogenesis in many literatures,[3–5] only several reports mentioned the efficacy of IFN in the suppression of tumor recurrence or in prolongation of survival period after ablation of HCC[6–12]. We once

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demonstrated the preventive activity of HCC recurrence by IFN-beta in a randomized controlled trial,[5] but intravenous type of IFN-beta was not universally available outside Japan in spite of the superiority of tumor suppressive activity to IFN-alpha.[13–17] Some investigators[14–17] showed that IFN acted as an anti-cancer agent in the treatment of HCC *in vivo* and *in vitro*. However, the actual efficacy of IFN in preventing recurrence of HCV-associated HCC in optimally treated patients remains unclear. Since some prospective study failed to demonstrate a beneficial effect of IFN-alpha in cumulative recurrence rate,[11] we analyzed a large cohort of patients for a long period up to 18 years.

To what extent IFN suppresses the recurrence rate of early stage of HCC, we analyzed a large retrospective cohort with and without a long-term administration of IFN-alpha in patients with HCC. The purposes of this study were (i) to evaluate the influence of IFN-alpha on HCC recurrence rate after treatment of an early stage of HCV-related HCC, and (ii) to explore effective ways of IFN administration, if any.

PATIENTS AND METHODS

Study population

A TOTAL OF 729 patients were diagnosed as having HCC associated with HCV-related chronic liver disease from 1990 to 2006 in our hospital. Among them, 379 patients underwent surgical resection or sufficient medical ablation therapy for small HCC (multiple up to three nodules, 3 cm or less each). All were positive for anti-hepatitis C antibody and negative for hepatitis B surface antigen. The consecutive patients were analyzed, who met inclusion criteria of (1) initial diagnosis of HCC (2) early stage of HCC (multiple up to three nodules, 3 cm or less each) (3) potentially curative manner of resection or radiofrequency ablation for HCC, and (4) positive HCV RNA. Exclusion criteria of this study were (1) positive portal vein invasion on imaging of computerized tomography or ultrasonography (2) residual HCC on imaging diagnosis after surgical or medical therapy (3) Child-Pugh score C (4) other etiology of liver disease (hepatitis B, alcoholic, non-alcoholic liver disease, etc.) (5) use of other anti-viral agents including interferon-beta (6) use of retinoid derivatives, and (7) concomitant malignant tumor in addition to HCC.

The diagnosis of HCC was established by integrated imagings of ultrasonography, dynamic computerized tomography (CT), magnetic resonance imaging (MRI). To exclude additional small HCC nodules in the liver, computerized tomographic hepatic arteriography (CT-HA) and computerized tomographic arterioportography (CT-AP) were also performed in 356 patients (93.9%). Among the consecutive 379 patients with surgical resection or sufficient radiofrequency ablation for HCC, 77 (20.3%) patients received intermittent IFN-alpha injection two or three times a week for 6 months or longer, mainly after the year of 1995 when this medication became available for use in Japan: Two (3.4%) of 59 patients received IFN therapy during 1990–1994, 21 (21.2%) of 99 patients during 1995–2000, and 54 (24.2%) of 223 patients during 2001–2006, respectively. The other 302 patients did not receive IFN therapy or other anti-viral therapy. None of the patients received any other anti-viral or anti-carcinogenic treatment including nucleoside analogues. We therefore, performed this analytical study as a retrospective cohort study.

Clinical background and laboratory data

Table 1 summarizes the profiles and laboratory data of the IFN group (group A) and the untreated group (group B) at the time of diagnosis of HCC. The median age in the IFN group was lower than that of the untreated group by 3 years, but the other features were not different between the two groups regarding demography, liver function, state of HCC, and treatment of HCC.

Interferon treatment and judgment of the effect

Seventy-seven patients underwent IFN therapy after treatment of HCC. IFN therapy was usually initiated within several months after ablation of HCC, and a median period from HCC treatment to initiation of IFN was 5.6 months.

All the patients received IFN-alpha (natural or recombinant): Seven received interferon plus ribavirin combination therapy, and 68 underwent interferon monotherapy. Ten patients (13.0%) underwent interferon therapy for 6 months or less, 15 patients (19.5%) for 7 to 12 months, 13 patients (16.8%) for 13 to 24 months, 28 (36.4%) for 25 to 60 months, and the remaining 11 (14.3%) for a prolonged period of 61 months or longer. As a whole, a median dose of 242 million units was administered during the median period of 24.2 months. A total of 50.6% of all the patients received IFN for 2 years or longer.

Judgment of IFN effect was classified according to elimination of HCV RNA and alanine aminotransferase (ALT) value at a time of 6 months after the end of the

Table 1 Profiles and laboratory tests of the patients with and without interferon

Groups/characteristic	Group A (interferon)	Group B (none)	*P**
Patients characteristics			
N	77	302	
Age (year) (median, range)	63 (43–77)	66 (39–87)	0.003
Sex (Male/Female)	46/31	191/111	0.57
Positive HBs antigen	0	0	NS
Positive HCV antibody	77 (100%)	302 (100%)	NS
Positive HCV-RNA	77 (100%)	302 (100%)	NS
Cancer characteristics before treatment			
Number of nodules			0.89
Solitary	63	260	
Two	11	33	
Three	3	9	
Size of maximal tumor (median, range)	18 (5–30)	18 (8–30)	0.50
Vascular invasion on imaging	0	0	NS
Cancer therapy			
Surgery	35 (45.5%)	146 (48.3%)	0.65
Radiofrequency ablation	42 (54.5%)	156 (51.7%)	
Laboraory findings (median, range)			
Albumin (g/dl)	3.6 (2.4–4.3)	3.6 (2.4–4.5)	0.80
Bilirubin (mg/dl)	1.0 (0.3–2.5)	1.0 (0.2–3.3)	0.96
Aspartic transaminase (IU)	54 (16–311)	54.5 (13–191)	0.94
Alanine transaminase (IU)	57 (12–273)	54 (11–230)	0.89
Platelet (×1000/cmm)	100 (20–272)	110 (20–256)	0.85
ICG R15 (%)	25 (1–75)	27 (2–78)	0.58
Alpha-fetoprotein (mg/L)	22 (3–1411)	22 (1–4950)	0.28
DCP (AU/L)	19 (11–635)	17 (0–1470)	0.50

*Non-parametric test (χ^2 test or Mann–Whitney *U*-test). DCP, des-gamma-carboxyprothrombin; ICG R15, indocyanine green retention test at 15 minutes.

treatment. Sustained virological response (SVR) was defined as persistent disappearance of HCV RNA after therapy, biochemical response (BR) as normal ALT values (40 IU/L or less) without elimination of HCV RNA for at least 6 months after therapy, and no response (NR) as persistently abnormal or only transient normalization of ALT for less than 6 months.

Follow-up and diagnosis of HCC

Physicians examined the patients every 4 weeks after entry to the study. Liver function tests and hematologic and virologic tests were conducted every month. To diagnose recurrent HCC nodules at an early stage, imaging studies were performed every 3 months, using ultrasonography and computerized tomography. Alfa-fetoprotein and des-gamma-carboxyprothrombin were also assayed bimonthly. When angiography demonstrated a characteristic hypervascular nodule, it was usually a specific finding for HCC in these follow-up patients, and histological confirmation was usually not required in the majority of these HCC patients. Most of the "angiographically-diagnosed HCC" showed intrahepatic multiplicity and pathognomonic findings of capsule formation or nodule-in-nodule appearance, or even portal vein invasion. If angiography did not show any hypervascular stain in a small hepatic nodule, histological study was always performed.

A total of 8 patients could not continue the IFN treatment due to side effects, following studies of tumor recurrence and survival were analyzed on an intention-to-treat basis.

Eight patients were lost to follow-up: 2 in IFN group and 6 in untreated group. Treated and untreated patients were followed at intervals of one month for a median observation period of 4.6 years, ranged from 0.1 to 18.4 years: 5.6 years in interferon group and 4.2 years in untreated group. The date of the last follow-up for this study was 30th August, 2009.

The end point of the study was tumor recurrence after treatment.