ういった防御機構をすりぬけて若年発がんが起こるものと考えられる．

このように，たとえ治療の必要がなくてもHBVキャリアの経過観察は専門的な知識が必要とされる．

おわりに

B型肝がん発がん抑止のためには，HBVキャリアがどの病期にいるかを診断することが肝要である．当科が提唱したこのHBステージ分類はその診断に有用と考える．治療適応例には早期に適切な抗ウイルス治療を開始し，肝病変の進展や肝発がん例を1名でも減少させたいと考えている．

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＜原　著＞

初発肝細胞癌に対するラジオ波焼灼療法後の異所再発に関する予測因子の検討

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要旨：初発肝細胞癌に対するラジオ波焼灼療法（RFA）による根治術後の異所再発に関する予測因子を検討した．当科にてRFAを用いて根治術を施行し，再発予防治療を施行せず，局所再発なく経過観察できた初発肝細胞癌患者56例を対象とした．17例で異所再発を認め，累積異所再発率は1年で12.0%，2年で36.3%，3年で53.5%であった．腫瘍多発群は単発群に比し4.78倍（$p=0.01$），血小板値$10.0\times10^4/\mu l$未満群は$10.0\times10^4/\mu l$以上群に比し5.25倍（$p<0.01$）再発率が高かった．報告されている外科的切除後の異所再発率と差がなく，外科的切除でも血小板低値群や腫瘍多発群は高異所再発群として報告されていることを考えると，再発予防治療が確立されるまでは，安全にRFAにて根治可能な病変であれば残存肝機能の観点から血小板低値群や腫瘍多発群はRFAを積極的に選択されるべきではないかと考えられた．

索引用語：　肝細胞癌　ラジオ波焼灼療法（RFA）　異所再発　予測因子　血小板値

背　景

肝細胞癌に対する治療法として1990年代になりラジオ波焼灼療法（RFA）が出現し[1)~4)]，経皮的エタノール注入療法に比し局所制御能の改善がみられるようになったとの報告がみられる[5)6)]．手技の簡便性も加わり，現在は肝細胞癌に対する局所療法として広く行われつつある．2005年に出版された肝癌診療ガイドライン[7)]においても，肝障害度A，Bであり単発の肝細胞癌や，3cm以下2，3個の肝細胞癌は外科的切除もしくは局所療法を選択すべきとされた．そのためRFAでも根治可能な肝細胞癌に対して，外科的切除と，RFAによる局所療法のどちらの治療法が望ましいのか判断に迷う症例が数多く存在する．

肝細胞癌の多くは慢性肝炎・肝硬変を背景肝としており，たとえ外科的切除などにて原発癌に対する治療を充分に実施できたとしても多中心性発癌による再発率が高く，肝細胞癌患者の予後不良の原因となっている．再発予防法の確立されていない現在においては，RFAにて局所療法を充分に施行できた症例においても，異所再発が高率に認められる．外科的切除による治療後の再発予測因子に関する検討は今までに多数報告されている[8)~12)]．一方，RFAによる治療後の異所再発予測因子に関しては，肝炎の成因がC型肝炎ウィルスであることや腫瘍多発症例などを挙げている報告[13)14)]が見られるが，現時点では未だ少ない．

RFAによる治療と外科的切除による治療とで異所再発率に大きな差がなく，RFAによる治療後の異所再発予測因子が，外科的切除による治療後の異所再発予測因子として報告されているものであれば，共通する予測因子を用いてRFAにて根治可能な病変の最適な治療法を選択することができるのではないかと考える．今回我々はRFAにて根治可能であった肝細胞癌に関する異所再発の予測因子を明らかにすることを目的とした．

対象と方法

2001年4月から2004年9月までの間に当科にてRFAを施行した初発の肝細胞癌患者のうち，治療終了時す

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Table 1 Characteristics of patients (n = 56)

Factor	
Age (years)	70.7±8.4 (38-84)
Sex (male：female)	40：16
Etiology (HBV：HCV：Other)	7：45：4
Stage† (Ⅰ：Ⅱ：Ⅲ)	33：18：5
Maximum tumor size (mm)	21.5±8.0 (9-45)
Number of tumor nodules (1：2：3)	48：7：1
Early enhancement†† (+：−)	44：12†††
AFP (ng/ml)	104.6±261.3
PIVKA-Ⅱ (mAU/ml)	82.3±148.3††††
Child-Pugh Classification (A：B：C)	44：10：2
Ascites (+：−)	2：54
Alb (g/dl)	3.7±0.6
T-Bil (mg/dl)	1.0±0.5
PT (%)	81.8±12.7††††
Platelet ($\times 10^4/\mu l$)	10.6±4.9
AST (IU/L)	67±37
ALT (IU/L)	59±42
RFA with TAE (+：−)	37：19

† The TNM staging system by Liver Cancer Study Group of Japan
†† Early enhancement on dynamic enhanced CT or dynamic enhanced MRI
††† One patient was diagnosed by artery angiography, and the other 11 patients were diagnosed by histology which showed well differentiated HCC.
†††† n = 54；Two patients taking warfarin were excluded

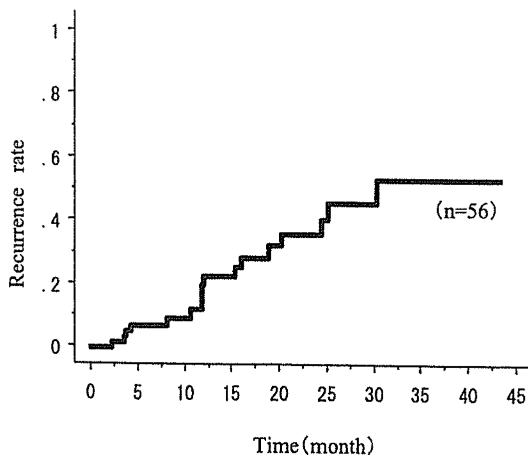

Fig. 1 Cumulative rate of intrahepatic distant recurrence in patients with primary HCC treated with RFA

べての肝細胞癌が焼灼されたと画像検査上判断でき3カ月以上経過観察ができた症例は64例であった．このうち6例はインターフェロン治療などの再発予防治療がその後施行されており，また，2例は初回の再発として局所再発を認めたためこれらの8例は今回の検討対象から除外し，計56例につき検討を行った．解析は2005年1月の時点で行った．なお，対象患者の患者背景は男性40例，女性16例，平均年齢70.7±8.4歳（38—84），肝炎の成因として肝炎ウィルスマーカーはHCV抗体陽性45例，HBs抗原陽性7例，両者陰性4例，両者陽性0例であった．また，平均腫瘍径21.5±8.0mm（9—45 mm），観察期間中央値16.4カ月（3—44カ月）であった（Table 1）．

今回ダイナミック造影CT，あるいはダイナミック造影MRIにて肝内に腫瘍早期濃染を認め，典型的な肝細胞癌の画像所見を認めたもの，腹部血管造影検査にて腫瘍濃染を認めたもの，もしくはエコーガイド下狙撃組織診を行い病理組織により肝細胞癌組織を確認できたものを肝細胞癌とした．RFA後は1～2カ月ごとに腫瘍マーカー（血清α-fetoprotein（AFP），血清protein induced by vitamin K absence or antagonist-II (PIVKA-II)）の測定を行い，また，約3カ月ごとに腹部超音波検査，ダイナミック造影CT検査，あるいはダイ

Table 2 Risk factors of intrahepatic distant recurrence in patients with primary HCC by univariate analysis

Variable		n	P value
Age (years)	< 70/ ≧ 70	22/34	0.08
Sex	Male/female	40/16	0.39
Etiology	HCV/HBV or Other	45/11	0.30
Stage†	I/II or III	33/23	0.63
Maximum tumor size (mm)	≦ 20/ > 20	36/20	0.40
Number of tumor nodules	1/ ≧ 2	48/8	0.005
Early enhancement††	+ / −	44/12	0.30
AFP (ng/m*l*)	< 20/ ≧ 20	30/26	0.13
PIVKA-II (mAU/m*l*)	< 40/ ≧ 40	31/23	0.08
Child-Pugh Classification	A/B or C	44/12	0.45
Alb (g/d*l*)	< 3.5/ ≧ 3.5	18/38	0.66
T-Bil (mg/d*l*)	< 1.5/ ≧ 1.5	48/8	0.41
PT (%)	< 80/ ≧ 80	20/34	0.74
Platelet ($\times 10^4/\mu l$)	< 10.0/ ≧ 10.0	30/26	0.02
AST (IU/L)	≦ 40/ > 40	16/40	0.84
ALT (IU/L)	≦ 40/ > 40	26/30	0.91
RFA with TAE	+ / −	37/19	0.82

†The TNM staging system by Liver Cancer Study Group of Japan
††Early enhancement on dynamic enhanced CT or dynamic enhanced MRI

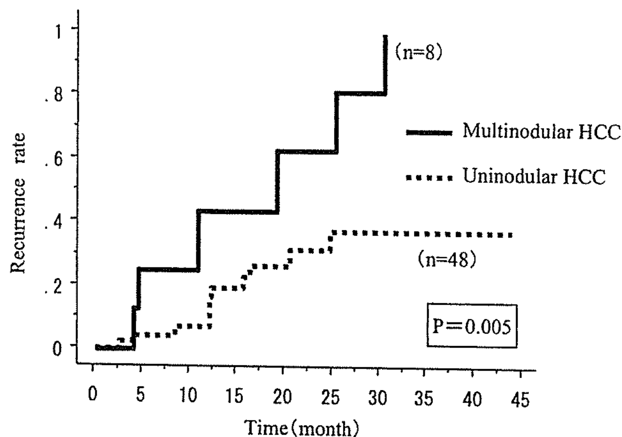

Fig. 2 Cumulative rate of intrahepatic distant recurrence in patients with primary multinodular HCC and those with primary uninodular HCC before RFA

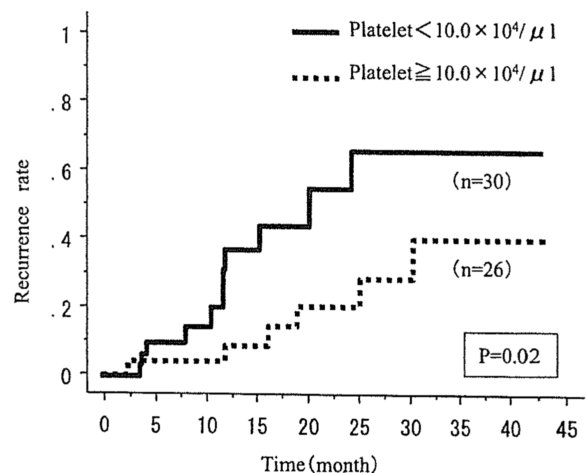

Fig. 3 Cumulative rate of intrahepatic distant recurrence in patients whose platelet count was less than $10.0 \times 10^4/\mu l$ and those whose platelet count was $10.0 \times 10^4/\mu l$ and over before RFA

ナミック造影MRI検査にて再発の有無についてフォローアップした．また，経過観察中にRFAにて焼灼した領域に隣接して再発する肝細胞癌を局所再発，それ以外の再発を異所再発とした．

患者背景因子として年齢，性別，肝炎の成因を，腫瘍因子としてStage（TNM分類），最大腫瘍径，個数，ダイナミック造影CTあるいはMRIでの腫瘍早期濃染の有無，AFP値，PIVKA-II値を，背景肝機能因子としてChild-Pugh分類，血清アルブミン（Alb）値，総ビリルビン（T-Bil）値，プロトロンビン時間（PT）値，血小板値，血清aspartate aminotransferase（AST）値，血清alanine aminotransferase（ALT）値を，治療因子として選択的肝動脈塞栓術（TAE）併用の有無につい

Table 3 Risk factors of intrahepatic distant recurrence in patients with primary HCC by multivariate analysis using the Cox proportional hazard model（n = 54）

Factor	P value	Hazard ratio	95% Confidence
Age（years） ≧70/＜70	0.16	2.36	0.72-7.75
Number of tumor nodules ≧2/1	0.01	4.78	1.41-16.13
AFP（ng/ml） ≧20/＜20	0.90	1.07	0.72-7.75
PIVKA-II（mAU/ml） ≧40/＜40	0.18	2.12	0.70-6.41
Platelet（×10⁴/μl） ＜10.0/≧10.0	0.009	5.25	1.52-18.06

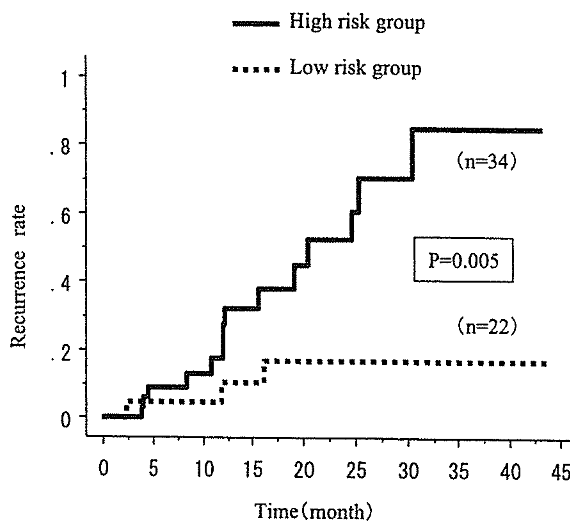

Fig. 4 Cumulative rate of intrahepatic distant recurrence in patients in a high risk group and those in a low risk group (patients in a high risk group；Patients either showed multinodular HCC or whose platelet count was less than $10.0\times10^4/\mu l$ before RFA, patients in a low risk group；Patients showed uninodular HCC and whose platelet count was $10.0\times10^4/\mu l$ and over before RFA)

て検討した．なお，Stage（TNM 分類）は原発性肝癌取り扱い規約第4版に従った．

焼灼はRITA社ないしRadionics社製穿刺針を使用し，持続的焼灼ないし段階的焼灼法にて施行した．約1週間後にダイナミック造影CTにて効果判定を行い，治療前のCTと比較するなどして焼灼範囲が少なくとも腫瘍のすべてを覆っていると判断できるまでRFAを繰り返し施行した．なお，腫瘍早期濃染がある肝細胞癌に対しては，可能な限りRFA施行前にTAEを施行した．

解析はStat view5.0®日本語版（HULINKS.Inc.）を用いて行った．再発率はKaplan-Meier法を用いて解析し，log-rank testによる検定を行った．また，単変量解析にて$p<0.15$であった因子に対して比例ハザードモデルを用いて多変量解析を行った．統計学的有意差検定はt検定，χ^2検定を行った．なお，有意水準は危険率5%とした．

結　果

56例中17例で異所再発を認めた．その累積再発率はKaplan-Meier法にて検討すると，1年12.0%，2年36.3%，3年53.5%であった（Fig. 1）．

年齢，性別，肝炎の成因，Stage（TNM分類），最大腫瘍径，個数，腫瘍早期濃染の有無，AFP値，PIVKA-II値，Child-Pugh分類，Alb値，T-Bil値，PT値，血小板値，AST値，ALT値，TAE併用の有無の17項目について，異所再発の発生に関してlog-rank testによる検定を行った結果，腫瘍多発群（$p<0.01$），血小板値$10.0\times10^4/\mu l$未満群（$p=0.02$）が高再発群として検出された（Table 2）（Fig. 2，3）．また，高年齢群（$p=0.08$），PIVKA-II高値群（$p=0.08$）において再発率が高い傾向を認めた（Table 2）．

単変量解析にて$p<0.15$であった腫瘍個数，血小板値，年齢，PIVKA-II値，AFP値の5因子に対して比例ハザードモデルを用いて多変量解析を行った（Table 3）．その結果，治療時腫瘍多発群は単発群に比し4.78倍再発率が高く（$p=0.01$），血小板値10.0万/μl未満群は，10.0万/μl以上群に比し5.25倍再発率が高い（$p<0.01$）ことが明らかとなった．

腫瘍個数，血小板値の2因子が独立因子であったことから，腫瘍単発かつ血小板値$10.0\times10^4/\mu l$以上であった群をLow risk group：（a）群，腫瘍多発もしくは血小板値$10.0\times10^4/\mu l$未満であった群をHigh risk group：

Table 4 Characteristics of patients in a high risk group and those in a low risk group

Factor	High risk group (n = 34)	Low risk group (n = 22)	P value
Age (years)	71.0±8.5	70.3±8.5	0.77
Sex (male：female)	22：12	18：4	0.17
Etiology (HBV：HCV：Other)	4：28：2	3：17：2	0.87
Stage † (Ⅰ：Ⅱ：Ⅲ)	19：10：5	14：8：0	0.17
Maximum tumor size (mm)	22.4±8.4	20.1±7.3	0.28
Early enhancement†† (+：−)	27：7	17：5	0.85
AFP (ng/ml)	106.5±271.0	101.7±251.8	0.95
PIVKA-Ⅱ (mAU/ml)	68.1±118.9 †††	103.1±184.0	0.40
Child-Pugh Classification (A：B：C)	25：7：2	19：3：0	0.40
Alb (g/dl)	3.6±0.6	3.9±0.6	0.03
T-Bil (mg/dl)	1.1±0.5	0.9±0.3	0.24
PT (%)	77.8±12.3 †††	87.6±11.1	0.004
AST (IU/L)	74±37	55±34	0.06
ALT (IU/L)	65±43	51±40	0.25
RFA with TAE (+：−)	21：13	16：6	0.40

high risk group：Patients either showed multinodular HCC or whose platelet count was less than $10.0\times10^4/\mu l$ before RFA
low risk group：Patients showed uninodular HCC and whose platelet count was $10.0\times10^4/\mu l$ and over before RFA
†The TNM staging system by Liver Cancer Study Group of Japan
††Early enhancement on dynamic enhanced CT or dynamic enhanced MRI
††n = 32；Two patients taking warfarin were excluded

(b) 群とすると，(a) 群では1年再発率 4.5%，2年再発率 16.9%，3年再発率 16.9% に対して，(b) 群では1年再発率 17.4%，2年再発率 52.5%，3年再発率 85.2% と有意に高値であった(p=0.005) (Fig. 4)．なお，両群間の背景因子には，肝機能因子 (Alb 値，PT 値) 以外に有意な差を認めなかった (Table 4)．

考 案

RFA は肝腫瘍に対する局所療法の一つとして 1990 年ごろより欧米で紹介され[1)2)]，本邦では 1999 年以降肝細胞癌の治療法の一つとして広く導入されるようになった[3)4)]．肝細胞癌に対する RFA 後の局所再発率に関して，Tateishi らは2年以降の累積局所再発率 2.4%[15)]，Curley らは平均観察期間 19 カ月で 3.6%[16)]，Rossi らは平均観察期間 23 カ月で 5%[17)] と報告しており，RFA による局所療法としての治療成績は良好なものである．当科の局所再発率も平均観察期間 16 カ月で 3.4% でありこれらの報告と同等であった．一方，肝細胞癌においては局所治療が良好であり局所再発を認めなくとも，多中心性発癌または肝内転移による異所再発が高率に認められる．実際，日本肝癌研究会の第 16 回全国原発性肝癌追跡調査報告によると，2年間で肝細胞癌の全治療症例の 31.7% が再発しており，そのうち 65.1% が異所再発であった[18)]．さらに，日本肝癌研究会は，肝細胞癌患者の 62.6% が高発癌群である肝硬変を背景肝として有しており，また，71.8% が HCV 抗体陽性であったと報告している[18)]ことから，肝硬変や，C 型肝炎ウィルスを肝炎の成因としている患者が多くを占め，そのため多中心性発癌が高頻度に発生していると考えられる．今回我々は，RFA 根治可能病変に対する最適な治療の選択指標を見出すために，RFA による治療後いかなる症例において異所再発が多く認められるのかを検討し，異所再発の予測因子を明らかにしようとした．そのため，再発時の肝細胞癌では，初発時の治療が異所再発の発見に対して影響を及ぼし得ると考え，初発の肝細胞癌患者のみを対象とした．また，局所再発が認められれば，局所再発に対する治療が実施され，その時点で画像上明らかでない異所再発に対する治療も TAE などにて行われる可能性があるため，局所再発を認めた症例も検討対象から除外した．

まず，今回我々の検討にて血小板値が低ければ異所再発を高率に認めるとの結果を得たが，血小板値のカットオフ値を $10.0\times10^4/\mu l$ と設定した．Ono らは血小板値が低ければ，それだけ肝の線維化が進行しており，血小板値 $10.0\times10^4/\mu l$ 以下では肝線維化度の F4 にあたる

症例が多いと報告している[19]．さらにKuboらは肝線維化が進むにつれC型慢性肝疾患では多中心性発癌を起こしやすいと報告している[20]．今回の検討では日本の肝細胞癌の発生における背景肝病変の現状とほぼ同じように[18]，約8割（45例/56例）という多くの症例がC型慢性肝疾患を背景肝病変としていることから，今回我々の検討にて血小板値が低い症例群において異所再発が多かった理由として，この群で線維化の進んだC型慢性肝疾患症例が多く存在し，非癌部の発癌ポテンシャルが高く，多中心性発癌による再発が高頻度にみられたためではないかと推測される．

また，腫瘍個数が複数であれば異所再発を高率に認めるとの結果を得た．これは同時期に多数の腫瘍が発生したものであれば，それだけ背景肝の発癌リスクが高く，多中心性発癌が起こりやすかったという可能性が考えられる．また，肝内転移の危険性は腫瘍個数が多いほど高くなり，異所再発率も高くなると推測される．さらに初発時に認められた複数の腫瘍が原発と転移巣という関係であれば，すでに肝内転移を起こしている状態であり，その時点で画像上描出できない肝内転移が他部位にも発生してしまっている可能性は非常に高く，そのため異所再発率が高くなってしまった可能性も考えられる．

以上を考え合わせると，肝癌の異所再発形式は多中心性発癌と肝内転移の2つがあるが，血小板低値例は多中心性発癌のリスクが高いため，また腫瘍多発例は多中心性発癌及び肝内転移の両者のリスクが高いため，結果として異所再発リスクが高いという結果が得られたのではないかと考える．なお，この結果は肝炎の成因がC型肝炎ウィルスである症例や多発例がマイクロ波凝固療法やRFA後の高異所再発群であったというIzumiらの報告[13]や，C型肝炎患者ではRFA後の異所再発症例において有意に多発例や血小板低値例が多く，腫瘍多発がRFA後の異所再発予測因子であったとのYamanakaらの報告[14]と相違しない結果であった．

今回の検討ではRFAによる治療後，年率約20%の異所再発を認めた．これはマイクロ波凝固療法やRFA後1年間で18%の異所再発を認めたとのIzumiらの報告[13]や，RFA後1年間で20.4%の異所＋局所再発を認めた（局所再発は1年で1.3%）とのTateishiらの報告[15]と同等の結果であった．また，外科的切除を施行した場合，1年間で18.3%の異所再発を認めたとのIkedaらの報告[21]や，左近[22]やShimada[8]らが外科的切除後1年間で約20%弱の異所再発を認めたと報告しているのとも同等の異所再発率であった．このことはRFAにて充分治療できる大きさの肝細胞癌であれば，RFAは外科的切除と同等の治療効果をあげることができることを示唆している．一方，外科的切除，RFAいずれの治療法を選択したとしても，このように高率に発生する異所再発のため肝細胞癌患者の予後の改善には限界が認められているのが現状である．

再発予測因子に関して，Shimadaらは肝細胞癌の外科的切除後では血小板低値群，Alb低値群，糖尿病合併群，γグロブリン高値群，AFP高値群，未分化癌群，静脈浸潤群において再発率が高いと報告しており[8]，Kuboらは高分化肝細胞癌の外科的切除後では，血小板低値群，有輸血歴群において再発率が高いと報告している[9]．このように外科的切除後においても今回の我々の結果と同様に血小板低値群が高再発群であるとの報告が見受けられる．さらに，Arii[10]やCha[11]らは外科的切除後の再発に関しては腫瘍因子で最大腫瘍径や個数が再発予測因子として認められると報告しており，外科的切除後においても腫瘍個数は重要な再発の予測因子となっている．今回我々の検討では，最大腫瘍径やダイナミック造影CTやMRIでの腫瘍早期濃染を指標とした分化度では再発危険度に差を認めなかった．外科的切除で最大腫瘍径を予測因子としている報告の患者背景をみると，一般的にRFAの適応とはなりにくい最大腫瘍径が50mmを超えている症例が多く含まれていることから，検討対象の患者背景が大きく異なっていたことが本検討で最大腫瘍径が予測因子とならなかった原因ではないかと思われる．分化度に関しては，RFAにて根治可能と判断される病期段階では異所再発率（肝内転移）に大きく寄与しない可能性もあるが，今回我々は早期濃染を認めた肝細胞癌，つまり分化度の低い肝細胞癌に対しては可能な限りTAEを併用していたことも，有意差が出なかった理由の一つとして考えられる．この点に関しては今後の更なる検討が必要である．

本検討により，RFA治療後では治療時単発かつ血小板値$10.0 \times 10^4/\mu l$以上のLow risk group：(a)群の3年異所再発率が16.9%と少ないのに対して，それ以外のHigh risk group：(b)群では3年異所再発率が85.2%と非常に高いことが明らかになった．RFAによる局所再発率が数%前後であり，局所再発が（a）群（b）群同率で発生すると仮定すると，（a）群の再発における局所再発の占める比率は極めて高く，逆に(b)群の再発における局所再発の占める割合は相対的に低下する．日本肝癌研究会が第16回全国原発性肝癌追跡調査報告

にて外科的切除率は初発時31.3%であるのに対し，再発時では2.2%しか外科的切除が行われていなかったと報告している[18]ように，肝予備能などの問題から，肝細胞癌を2度以上外科的切除することはまれである．RFA治療後の異所再発率と，外科的切除後の異所再発率が大きく違わないこと，またRFA治療後の異所再発予測因子は外科的切除後の異所再発予測因子でもあることを考えれば，（b）群ではたとえ外科的切除をしたとしても数年で高率に再発し，その再発癌のほとんどに対して非外科的治療が施行されることとなる．さらに今回の検討で，（b）群は（a）群に比して有意にAlb値やPT値が低く，ALT値には差がなかったもののAST値が高い傾向を示していたことから，（b）群では（a）群に比して残存肝機能の低下した症例が多いものと考えられる．つまり高再発群は治療時の肝機能も低再発群に比し低下しており，高再発群を初発時に肝切除することは，肝予備能をさらに低下させ，数年以内に起こる可能性の極めて高い再発時の治療の選択肢を狭めてしまうのではないかと危惧される．今後生存率の検討は必要であるが，再発予防治療が確立されるまでは，多発例または血小板低値例において，RFA適応病変であればRFAを積極的に選択するべきではないかと考える．逆に，RFA治療後の局所再発率が限りなく0であると予測される病変でなければ，低再発群は手術を積極的に選択するべきではないかと考える．

腫瘍個数は血管内浸潤などと違い特殊な検査機材を必要とせず，血小板値は安価かつ簡便に測定できる検査データの一つである．C型慢性肝疾患を背景肝とする症例の多い日本では，これら2つの指標によりRFA適応病変の異所再発のリスクが今回の結果のように大きく分かれるということから，初発肝細胞癌に対してRFA治療すべきか外科的切除をすべきか悩む症例において治療法を選択する上で腫瘍個数と血小板値が重要な判断指標となり得ると考える．

結　語

当科における初発肝細胞癌患者に対するRFA後の異所再発に関して検討を行った．累積異所再発率は1年12.0%，2年36.3%，3年53.5%であり，報告されている外科的切除後の異所再発率と差はなかった．また，治療時腫瘍多発例，血小板値$10.0 \times 10^4/\mu l$未満例はRFA治療後の異所再発率が非常に高かった．治療時腫瘍多発例や血小板低値例は外科的切除でも異所再発率が非常に高いことから，再発予防治療が確立されるまでは，安全にRFAで根治可能な病変であればRFAを積極的に治療法として選択すべきではないかと考えられた．

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Predictive factors for intrahepatic distant recurrence of hepatocellular carcinoma after radiofrequency ablation for primary tumors

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We studied 56 patients with primary hepatocellular carcinoma (HCC) treated with radiofrequency ablation (RFA) in our hospital to clarify the predictive factors for intrahepatic distant recurrence. The observation period was 16.4 months. Cumulative rate of recurrence was 12.0% in a year and 53.5% in 3 years. Multivariate analysis revealed that multinodular HCC (odds ratio 4.78, $p=0.01$) and platelet count $<10.0\times10^4/\mu l$ (odds ratio 5.25, $p<0.01$) were considered to be useful predictive factors for recurrence. These recurrence rate and factors after RFA were similar to those after surgical resection. Therefore, patients showed uninodular HCC and whose platelet count was $10.0\times10^4/\mu l$ and over before treatment might not be treated with RFA, but surgical resection if possible. However, considering remnant liver function and recurrence rate, we think patients either showed multinodular HCC or whose platelet count was less than $10.0\times10^4/\mu l$ before treatment should be treated with RFA if possible.

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Usefulness of elastometry in evaluating the extents of liver fibrosis in hemophiliacs coinfected with hepatitis C virus and human immunodeficiency virus

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Abstract

The newly developed elastometer, FibroScan®, was utilized to evaluate liver fibrosis in hepatitis C virus (HCV)- and human immunodeficiency virus (HIV)-coinfected 33 hemophiliacs and HIV-uninfected 24 patients with chronic hepatitis C. Chronicity in the liver was categorized into 4 stages by abdominal ultrasound (AUS): 1 (normal or fatty liver); 2 (chronic liver disease, mild); 3 (moderate); and 4 (severe). Stiffness of the liver was significantly increased as AUS stages advanced: 5.4 ± 2.2 ($N = 3$) versus 7.5 ± 2.7 ($N = 9$), in stage 1; 4.9 ± 1.7 ($N = 2$) versus 9.9 ± 6.0 ($N = 10$), in stage 2, 13.5 ± 4.7 ($N = 5$) versus 12.9 ± 5.9 ($N = 6$), in stage 3, and 22.0 ± 9.5 ($N = 14$) versus 28.1 ± 21.3 ($N = 8$), in stage 4, in non-HIV group and in HIV group, respectively ($P = 0.004$ and 0.007). Stiffness was correlated with AUS stages ($r = 0.740$, $P < 0.001$), platelet counts (PLT; $r = -0.642$, $P = 0.001$) and 7S domain of type IV collagen (IV-coll; $r = 0.480$, $P = 0.024$) in non-HIV group, while in HIV group, with IV-coll ($r = 0.801$, $P < 0.001$), AUS stages ($r = -0.603$, $P < 0.001$), procollagen type III peptides (P-III-P; $r = 0.621$, $P = 0.001$), PLT ($r = -0.480$, $P = 0.005$), and hyaluronic acid ($r = 0.433$, $P = 0.027$). FibroScan® is absolutely noninvasive and can be the alternative to liver biopsy, especially in patients with bleeding tendency.

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Keywords: Liver fibrosis; HCV; HIV; Stiffness

1. Introduction

It has been well documented that coinfection of hepatitis C virus (HCV) and human immunodeficiency virus (HIV) might accelerate the progression of liver fibrosis, as compared with HCV infection alone [1]. However, especially in patients with hemophilia coinfected with HCV and HIV, it is practically difficult to perform liver biopsy, the established gold standard to evaluate the extents of liver fibrosis, because of their bleeding tendency. Moreover, tiny biopsied specimens, corresponding to only one forty thousandth of entire liver volume, may sometimes cause misleading explanations. Although rough estimation of liver fibrosis can be performed by abdominal ultrasound (AUS), it would be somewhat subjective and dependent on technical experience. In addition, several serum markers for liver fibrosis such as procollagen type III peptides (P-III-P), 7S domain of type IV collagen (IV-coll), and hyaluronic acid, have been commercially available, however, those substances might just reflect overproduction of collagen in vivo.

Recently, a new noninvasive device to quantify liver fibrosis, FibroScan®, was developed by Echosens (Paris, France). This device is based on one-dimensional transient elastography, using both ultrasound (5 MHz) and low-frequency (50 Hz) elastic waves, whose propagation velocity is directly proportional to elasticity [2]. Usefulness of this device to assess the extents of liver fibrosis in patients with chronic hepatitis was already reported from France [3], and from Japan [4]. In this study, we attempted to evaluate its usefulness

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in hemophilacs coinfected with HCV and HIV, in whom liver biopsy is ordinarily contraindicated, as well as in non-hemophiliacs infected by HCV alone.

2. Patients and methods

Hemophiliacs coinfected with HCV and HIV or non-hemophiliacs infected by HCV alone, who consulted Division of AIDS Research Center or Division of Gastroenterology, International Medical Center of Japan, from February to April, 2004, were randomly enrolled in this study, after informed consent was given. The patient received elastometry with FibroScan® version 2 (FibroScan® 502: Echosens, Paris, France), as previously reported [4], at the same time with B-mode AUS, by two hepatologists (N.M. and M.I.) with over 15-year experience. The elasticity of the liver was measured 10 times and the median value, which was automatically calculated, was considered as Stiffness (KPa). AUS findings of the liver were categorized into 4 stages, based on the extents of surface irregularity, dullness of the edge, heterogeneity of internal echogenecity, blurriness of hepatic veins, and imbalance in size of both lobes (hypertrophy of the caudate lobe and/or atrophy of the right lobe): (1) normal or fatty liver; (2) chronic liver disease, mild; (3) moderate; and (4) severe. On the same day, serum levels of albumin, alanine aminotransferase (ALT), and liver fibrosis markers (P-III-P, IV-coll, and hyaluronic acid), were measured. HCV genotype and peripheral platelet counts (PLT) were also determined. Only in HIV group, CD4 counts and HIV RNA concentrations were determined. The correlation between Stiffness and AUS stage, PLT, or serum liver fibrosis markers were investigated.

In patients, where the age of blood transfusion or the first infusion of clotting factor concentrates could be identified, annual progression rate of liver fibrosis (R-value: KPa/year) was arbitrarily calculated, according to the following equation. R-value = [Stiffness − 5.4]/[Age (at the elastometry) − Age (of the HCV infection)], where 5.4 is the mean value of Stiffness in patients with normal AUS finding (data shown in Section 3).

2.1. Statistical analysis

The data were shown as mean ± standard deviation. The correlation between the Stiffness and AUS stage was determined by Kruskal–Wallis test. The correlation between the parameters was determined by Pearson's correlation coefficient or Spearman's correlation coefficient. The comparisons between the data were analyzed by Mann–Whitney U-test or Fisher's exact test.

3. Results

Thirty-three hemophiliacs coinfected with HCV and HIV (HIV group; hemophilia A/B = 23 patients/10 patients; all males) and 24 non-hemophiliacs infected by HCV alone (non-HIV group; 12 males and 12 females), were enrolled in this study, and received B-mode AUS and elastmetry at the same time. The demographic features and laboratory data of both groups were shown in Table 1. All the patients in HIV group have been successfully treated by highly active antiretroviral therapy (HAART) since 1996, and in 23 patients, HIV RNA concentrations were maintained less than 50 copy/ml. Seven out of 21 (33%) patients in HIV group were infected by HCV with uncommon genotype in Japan (1a or 3a). The patients were classified into 4 stages on AUS: stage 1: 3 and 9; stage 2: 2 and 10; stage 3: 5 and 6; stage 4: 14 and 8, in non-HIV group and HIV group, respectively. Apparently, in non-HIV group, most of the patients were older and had more advanced stages of chronic liver disease, as compared with those in HIV group. Stiffness of the liver in each AUS stage was shown as follows: 5.4 ± 2.2 and 7.5 ± 2.7, in stage 1; 4.9 ± 1.7 and 9.9 ± 6.0, in stage 2, 13.5 ± 4.7 and 12.9 ± 5.9, in stage 3, 22.0 ± 9.5 and 28.1 ± 21.3, in stage 4,

Table 1
Demographic features and laboratory data of non-HIV group and HIV group

Parameters	Non-HIV group (N = 24)	HIV group (N = 33)	P-value
Age	69 ± 13	39 ± 11	0.000[a]
Gender (M/F)	12/12	33/0	0.000[b]
Albumin (g/dl)	4.0 ± 0.4	4.3 ± 0.3	0.004[a]
ALT (U/l)	60 ± 45	67 ± 54	NS[a]
PLT ($\times 10^4/\mu l$)	12.5 ± 5.2	19.0 ± 8.6	0.003[a]
CD4 counts (μl^{-1})	n.d.	442 ± 230	
P-III-P (U/ml)	1.2 ± 0.3	0.9 ± 0.3	0.003[a]
IV-coll (ng/ml)	8.3 ± 3.1	6.2 ± 2.7	0.013[a]
Hyaluronic acid (ng/ml)	335 ± 399	210 ± 190	NS[a]
HCV genotype (1/1a/1a + 1b/1b/2a/2b/3a/3a + 2b)	2/0/0/8/1/2/0/0	4/4/1/5/3/2/1/1	
AUS stage (1/2/3/4)	3/2/5/14	9/10/6/8	0.031[c]

n.d.: not determined, NS: not significant. The abbreviations used are: ALT, alanine aminotransferase; PLT, platelet counts; P-III-P, procollagen type III peptides; IV-coll, 7S domain of type IV collagen; AUS, abdominal ultrasound.
[a] Mann–Whitney U-test.
[b] Fisher's exact test.
[c] Pearson's χ^2-test.

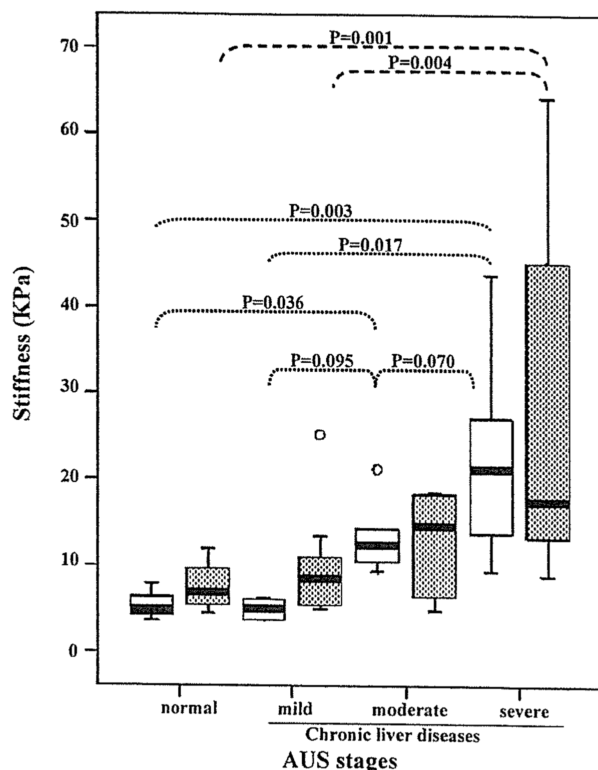

Fig. 1. Box plots of Stiffness for each AUS stage in non-HIV group and HIV group. The *top* and *bottom* of the boxes are the first and third quartiles, respectively. The length of the box represents the interquartile range within which 50% of the values were located. The *line* through the middle of each box represents the median. The *error bars* show the minimum and maximum values. Open boxes and dotted boxes show the data of non-HIV group and those of HIV group, respectively. The P-value was calculated by Mann–Whitney U-test.

in non-HIV group and in HIV group, respectively ($P = 0.004$ and 0.007, by Kruskal–Wallis test in each group). There was no significant difference between non-HIV group and HIV group, in each AUS stage. Box plots of Stiffness for each AUS stage were demonstrated in Fig. 1. In non-HIV group, there existed significant difference in Stiffness between stages 4 and 2 ($P = 0.017$), between stages 4 and 1 ($P = 0.003$), and between stages 3 and 1 ($P = 0.036$). In addition, there was marginal difference between stages 4 and 3 ($P = 0.070$), and between stages 3 and 2 ($P = 0.095$). While, in HIV group, Stiffness in stage 4 was significantly higher than that in stage 1 ($P = 0.001$), and that in stage 2 ($P = 0.004$).

To examine usefulness of elastometry in differentiating advanced stage of chronic liver disease from non-advanced one, similar analysis was conducted after patients of stages 2 and 3 were combined together. Chronicity in the liver was simply categorized into 3 modified AUS stages: normal (stage 1), non-advanced (stage 2 + 3) and advanced (stage 4). Stiffness of the liver in each modified AUS stage was shown as follows: 5.4 ± 2.2 ($N = 3$) and 7.5 ± 2.7 ($N = 9$), in stage 1 (normal), 11.1 ± 5.7 ($N = 7$) and 11.1 ± 6.0 ($N = 16$), in stage 2 + 3 (non-advanced), 22.0 ± 9.5 ($N = 14$) and 28.1 ± 21.3 ($N = 8$), in stage 4 (advanced), in non-HIV group and in HIV group, respectively ($P = 0.003$ and 0.004, by Kruskal–Wallis test in each group). Again, there was no significant difference between non-HIV group and HIV group, in each modified AUS stage. In addition, as compared with other liver fibrosis markers such as P-III-P, IV-coll, hyaluronic acid, and PLT, Stiffness was found to be the most useful in differentiating advanced stage of chronic liver disease from non-advanced one, both in non-HIV group and in HIV group (Fig. 2).

3.1. *Correlation between Stiffness and other parameters for liver fibrosis*

To evaluate usefulness of elastometry, we investigated correlation between Stiffness and conventional liver fibrosis markers such as P-III-P, IV-coll, and hyaluronic acid, including AUS stage and PLT. In non-HIV group, Stiffness was significantly correlated with AUS stage ($r = 0.740$, $P < 0.001$), PLT ($r = -0.642$, $P = 0.001$), and IV-coll ($r = 0.480$, $P = 0.024$). On the other hand, in HIV group, Stiffness was significantly correlated with IV-coll ($r = 0.801$, $P < 0.001$), AUS stage ($r = 0.603$, $P < 0.001$), P-III-P ($r = 0.621$, $P = 0.001$), PLT ($r = -0.480$, $P = 0.005$), and hyaluronic acid ($r = 0.433$, $P = 0.027$).

3.2. *Correlation between platelet counts and other parameters for liver fibrosis*

It has been well established that PLT are inversely proportional to the extents of liver fibrosis [5]. In this study, we investigated correlation between PLT and conventional liver fibrosis markers, AUS stage or Stiffness. In non-HIV group, PLT were significantly correlated with AUS stage ($r = -0.647$, $P = 0.001$), IV-coll ($r = -0.643$, $P = 0.001$), Stiffness ($r = -0.642$, $P = 0.001$), P-III-P ($r = -0.526$, $P = 0.012$), and hyaluronic acid ($r = -0.424$, $P = 0.049$). On the other hand, in HIV group, PLT were significantly correlated with Stiffness ($r = -0.480$, $P = 0.005$), AUS stage ($r = -0.415$, $P = 0.016$), and IV-coll ($r = -0.417$, $P = 0.031$).

3.3. *Annual progression rate of liver fibrosis*

We attempted to evaluate whether HIV coinfection may affect progression of liver fibrosis in patients with chronic hepatitis C. In non-HIV group, the age of blood transfusion was identified in 9 patients. Two of them had received interferon (IFN) therapy: IFN monotherapy and IFN-α2b/ribavirin, each, without sustained virological response (SVR). On the other hand, in HIV group, they had received multiple infusions of clotting factor concentrates, it was practically impossible to determine the time of the first infection of HIV or HCV. We speculated the age of the first infusion of such concentrates as the time of HCV infection, in 23 patients, in order to arbitrarily calculate annual progression rate of liver fibrosis (R-value). Twelve out of them had history of IFN therapy: IFN monotherapy, IFN-α2b/ribavirin and Pegylated IFN-α2a in 3, 5 and 4 patients,

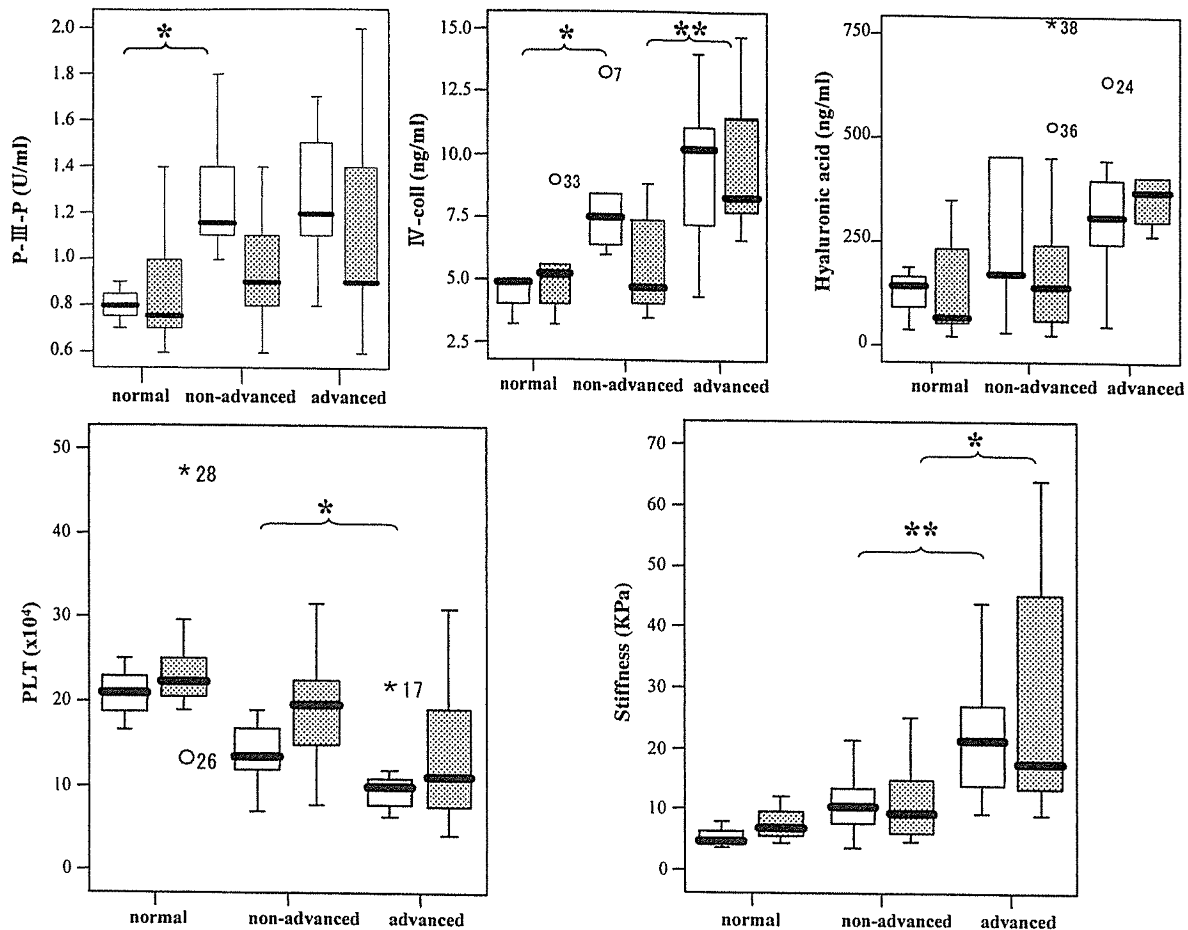

Fig. 2. Box plots of liver fibrosis markers (P-III-P, IV-coll, hyaluronic acid), PLT and Stiffness for each modified AUS stage in non-HIV group and HIV group. Open boxes and dotted boxes show the data of non-HIV group and those of HIV group, respectively. The P-value was calculated by Mann–Whitney U-test ($^{*}P<0.05$, $^{**}P<0.01$).

respectively. Four of them (33%) obtained SVR. There was no significant difference in R-value between non-HIV group and HIV group (0.32 ± 0.17 versus 0.38 ± 0.70; $P=0.125$, by Mann–Whitney U-test). In HIV group, there was no significant difference in R-value between IFN-treated ($N=12$) and untreated ($N=11$) patients (0.34 ± 0.50 versus 0.43 ± 0.90, $P=0.710$). Furthermore, in HIV group treated by IFN, there was no significant difference in R-value between patients with SVR ($N=4$, 0.08 ± 0.10) and those without SVR ($N=7$, 0.52 ± 0.60; $P=0.252$).

4. Discussion

In this study, we attempted to validate Stiffness measured by the newly developed elastometer, FibroScan® 502, in evaluating the extents of liver fibrosis in hemophiliacs coinfected with HIV and HCV (HIV group). Non-hemophiliacs infected by HCV alone (non-HIV group), could be regarded as a control, since hemophilia per se may not affect inflammation or fibrosis in the liver. It has been recently established that Stiffness is strongly correlated with liver fibrosis markers as well as F score evaluated by liver biopsy, in non-HIV group [3,4]. Liver biopsy could not be practically performed in clinical settings, however, such correlation was similarly, and even more significantly, confirmed also in HIV group. In this study, correlation between Stiffness and other parameters for liver fibrosis was rather weak in non-HIV group where more than half of the patients might have liver cirrhosis, probably because the variations of such liver fibrosis markers were greater in more advanced stage of chronic liver disease as shown in Fig. 2, as well as described previously [4]. Although AUS findings might be somewhat subjective, Stiffness in stage 4 was significantly higher than those in stages 1 and 2, both in HIV group and in non-HIV group, in this study (Fig. 1). In addition, when the patients of stages 2 and 3 were combined together, Stiffness was the only tool in differentiating advanced stage of chronic liver disease from non-advanced one, both in non-HIV group and in HIV group, as shown in Fig. 2. Accordingly, measurement of Stiffness could be a very useful tool to identify patients, at least, with advanced liver fibrosis. In contrast, PLT, which have been widely accepted as one of the very sensitive markers for liver fibrosis [5], seemed to be less reliable in HIV group than in

non-HIV group. Possibility of influence of HAART regimens on PLT counts should be further investigated in a future study.

It has been occasionally described that HIV coinfection increases the risk of cirrhosis and even hepatocellular carcinoma (HCC) in HCV-infected patients [6]. In addition, whether introduction of HAART or IFN therapy could affect these situations would be undoubtedly our major concern. Kramer et al. [7] recently reported that HIV coinfection accelerated liver fibrosis in pre-HAART era, but not in HAART era, while it did not affect the risk of HCC in HCV infected U.S. veterans in both eras. Similarly, Marine-Barjoan et al. [8] found that early HAART may slow liver fibrosis progression in coinfected French patients. On the contrary, Martinez-Sierra et al. [9] showed that the immune response to HAART did not influence liver fibrosis progression rate in coinfected Spanish patients. Moreover, it has been recently pointed out that HAART regimens including nevirapine, one of nonnucleoside reverse-transcriptase inhibitors, may be associated with faster liver fibrosis progression in coinfected patients [10], especially with advanced stages of liver fibrosis [11]. In this study, we arbitrarily calculated annual progression rate of liver fibrosis, using Stiffness and duration of HCV infection. Interestingly, there was no significant difference between non-HIV group and HIV group receiving HAART. This may be quite consistent with our previous findings that plasma levels of TGF-β and IL-13, known cytokines to play pivotal roles in liver fibrosis, were significantly decreased in coinfected patients under HAART, as compared with those in patients infected HCV alone (unpublished results). Furthermore, we could not get any definitive results that IFN therapy may counteract progression of liver fibrosis, because of the small numbers of patients examined and short terms of follow-up after IFN therapy. Further regular check-ups of the elasticity of the liver would be prerequisite to settle these controversial issues.

In conclusion, FibroScan® is absolutely noninvasive and can be the alternative to liver biopsy, especially in patients with bleeding tendency. It is strongly suggested that HIV/HCV coinfection may not accelerate liver fibrosis progression in HCV-related chronic liver disease, at least in HAART era.

Acknowledgements

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Survivin downregulation by siRNA sensitizes human hepatoma cells to TRAIL-induced apoptosis

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Abstract. Survivin, an anti-apoptotic protein, is abundantly expressed in a variety of cancer cells, including hepatoma cells, resulting in the resistance of these cells to various apoptotic stimuli. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is known to induce cancer cell-specific apoptosis, but hepatoma cells are resistant to TRAIL-induced apoptosis. In the present study, we have examined whether the downregulation of survivin by short interfering RNA (siRNA) promotes spontaneous or TRAIL-induced apoptosis in Huh-7 human hepatoma cells. Survivin siRNA transfection downregulated the expression of survivin in Huh-7 cells and reduced cell viability by 20% through inducing spontaneous apoptosis. TRAIL (1 to 2 ng/ml) only slightly induced apoptosis in Huh-7 cells; however, survivin siRNA transfection apparently enhanced TRAIL-induced apoptosis. These results suggest that the level of survivin is linked to the susceptibility of Huh-7 cells to TRAIL. It is possible that survivin downregulation by siRNA combined with TRAIL administration may provide a new therapeutic strategy against hepatoma.

Introduction

Survivin, the smallest mammalian member of the inhibitor of apoptosis (IAP) family, plays a key role, not only in cell division but also in apoptosis inhibition, by repressing caspase activities (1-3). Survivin is abundantly expressed in a variety of cancer cells, including hepatoma cells, but not in normal cells (3-6), resulting in the resistance of cancer cells to various apoptotic stimuli (3,5,7,8). In addition, the overexpression of survivin in cancer is closely related to unfavorable disease outcome (3,6,9-11). Therefore, survivin is a possible target of cancer therapy. In fact, the downregulation of survivin by anti-sense oligonucleotides, short interfering RNA (siRNA) and dominant-negative mutant induces apoptosis of cancer cells or sensitizes cancer cells to chemotherapeutic agents and irradiation (12).

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, induces apoptosis in a variety of cancer cells with little or no effect on normal cells (13,14). However, several cancer cells, including hepatoma cells, are resistant to TRAIL-induced apoptosis (15). Griffith *et al* have reported that the cellular level of survivin is linked to the resistance against TRAIL-induced apoptosis of renal cell carcinoma cells (16). It has been also reported that the downregulation of survivin by siRNA sensitizes resistant melanoma cells to TRAIL-induced apoptosis (17). Moreover, we previously reported that interferon (IFN)-α downregulated the survivin expression in human hepatoma cells and sensitized these cells to TRAIL-induced apoptosis, and ectopic expression of survivin partially rescued these cells from IFN-α TRAIL-induced apoptosis (18). Therefore, it is possible that survivin plays a role in the resistance to TRAIL-induced apoptosis in human hepatoma cells. To confirm this, in the present study, we examined whether downregulation of survivin by siRNA can sensitize human hepatoma cells to TRAIL-induced apoptosis.

Materials and methods

siRNAs. To select siRNA duplexes for survivin mRNA, we searched for sequences of AA and the N19 (N, any nucleotide) from the open reading frame of human survivin as described previously (19). siRNAs with the following sequences were used for experiments: surA, 5'-GGUUAAUUCUUCAAA CUGCTT-3' (antisense); surB, 5'-GCAAUUUUGUUCUUG GCUCTT-3' (antisense).

siRNA against green fluorescent protein (GFP) was also used as a control: siGFP, 5'-UGCGCUCCUGGACGUAGC CTT-3' (antisense). All siRNAs were purchased from Nihon Bioservice (Saitama, Japan).

Cell culture and transfection. Huh-7 human hepatoma cells were maintained in RPMI with 5% fetal bovine serum. Cells were plated on a 96-well plate or Lab-Tek chamber slide at

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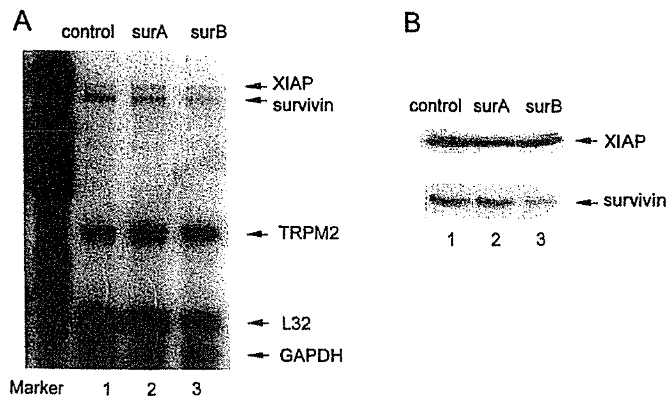

Figure 1. Effect of siRNA transfection on survivin expression in Huh-7 cells. Huh-7 cells were transfected with siGFP as a control (lane 1), surA (lane 2) or surB (lane 3), respectively. (A) The levels of indicated mRNAs were analyzed by RNase protection assay. (B) The expression of XIAP and survivin was analyzed by Western blotting. Results are from one representative experiment from a total of three performed.

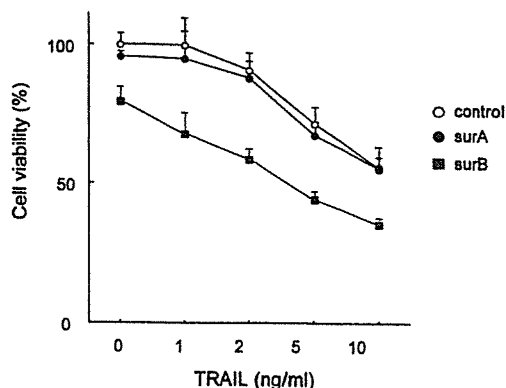

Figure 2. Effect of siRNA transfection with or without TRAIL on the viability of Huh-7 cells. Huh-7 cells were transfected with siGFP as a control (○), surA (●) or surB (■), respectively, and then treated with various concentrations of TRAIL for 12 h. Cell viability was determined by the colorimetric method. Data represent mean ± SD values of the four experiments.

an appropriate density. A day later, cells were transfected with each siRNA (4 pmol/10^4 cells) using oligofectamine (Gibco-Invitrogen, Rockville, MD, USA) according to the instructions provided by the manufacturer. The medium was replaced with fresh medium 6 h after transfection, and the cells were cultured for 36 h and treated with or without varying concentrations of TRAIL for 12 h.

RNase protection assay. The RNase protection assay was performed using a RiboQuant Multi-Probe RNase Protection Assay System (BD PharMingen, Franklin Lakes, NJ, USA). According to the instructions provided by the manufacturer, an hAPO5c (inhibitors of apoptosis) template set, including an L32 ribosomal protein and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) template as internal controls, was labeled with [α^{32}P]-UTP using T7 RNA polymerase. The labeled RNA probes were hybridized with 10 μg of total RNA from Huh-7 cells transfected with each siRNA. Samples were digested with RNase to remove single-stranded (non-hybridized) RNA. The remaining probes were resolved on 6% urea-polyacrylamide-bis-acrylamide gels. Gels were dried and analyzed using an image analyzer (BAS; Fuji Film Co., Tokyo, Japan).

Western blot analysis. Cells were lysed by adding lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% SDS, 1% NP40 and 100 μg/ml PMSF) for 10 min at 4°C and passed several times through a 25-gauge needle. The same amount of protein from each lysate (20 μg/well) was subjected to 12% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes that were then blocked for 1.5 h using 5% non-fat dried milk in PBS containing 0.1% Tween-20 (PBS-T), washed with PBS-T and incubated at room temperature for 1 h in the presence of each antibody (mouse monoclonal anti-human XIAP from MBL, Nagoya, Japan; and rabbit polyclonal anti-human survivin from Alpha Diagnostic International Inc., San Antonio, TX, USA). The membranes were washed with PBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG for 1 h. Following washing with PBS-T, immunoreactive bands were visualized using the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, UK).

Analysis of cell viability and apoptosis. Cell viability was determined by the colorimetric method using a Cell Counting kit (Wako Life Science, Osaka, Japan). The absorbance of each well was measured at 405 nm with a microtiter plate reader (Multiskan JX; Thermo BioAnalysis Co., Tokyo, Japan). For the detection of apoptosis, TUNEL assay was performed using a DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI, USA). Briefly, the cells cultured in Lab-Tek chamber slides were fixed in 4% paraformaldehyde in PBS. The fragmented DNA of apoptotic cells was detected by catalytically incorporating fluorescein-12-dUTP at 3'-OH DNA ends using the terminal deoxynucleotidyl transferase. 1 μg/ml of propidium iodide (PI) was added for nuclear staining. Sample slides were directly visualized by fluorescence microscopy (BX 50; Olympus, Tokyo, Japan) at 520 nm for the green fluorescence of fluorescein and at >620 nm for the red fluorescence of PI.

Results

siRNA transfection downregulates survivin expression. To confirm that siRNA transfection downregulates survivin expression in Huh-7 cells, RNase protection assay and Western blotting were performed (Fig. 1). surA transfection did not repress the level of survivin mRNA, but slightly upregulated the level of XIAP mRNA. In contrast, surB transfection repressed the level of survivin mRNA, but did not upregulate the level of XIAP mRNA (Fig. 1A). The levels of TRPM2, L32 and GAPDH mRNAs were almost unchanged by the transfection of surA and surB. Similarly, surB transfection clearly repressed survivin expression, but did not repress XIAP expression (Fig. 1B).

Survivin downregulation by siRNA enhances TRAIL-mediated cytotoxicity. We examined the effect of survivin siRNA transfection with or without TRAIL on cell viability (Fig. 2). surB transfection alone repressed the viability of Huh-7 cells by almost 20% compared with the control but surA transfection

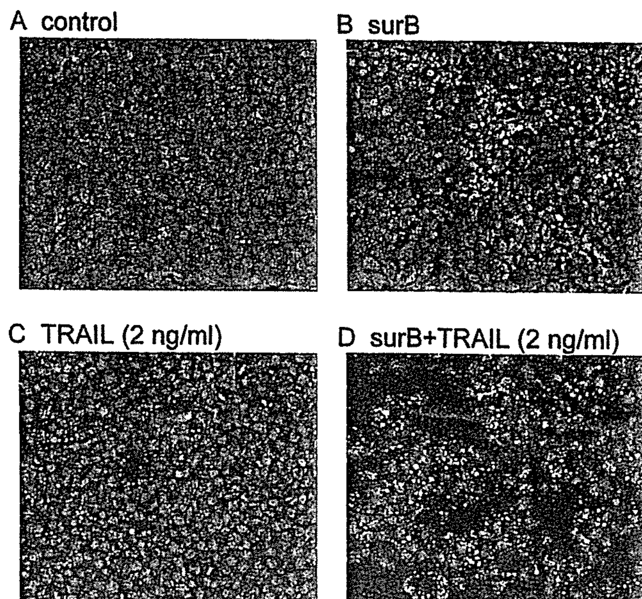

Figure 3. Effect of siRNA transfection with or without TRAIL on apoptosis in Huh-7 cells. Huh-7 cells were transfected with (A) siGFP alone as a control, (B) suB alone, (C) siGFP followed by TRAIL (2 ng/ml) treatment and (D) surB followed by TRAIL (2 ng/ml) treatment. Apoptotic cells were detected by TUNEL assay. Results are from one representative experiment from a total of four performed.

did not. Low concentrations of TRAIL (1 and 2 ng/ml) showed little effect on the viability of control cells, although high concentrations of TRAIL (5 and 10 ng/ml) reduced the viability. On the other hand, surB transfection sensitized Huh-7 cells to TRAIL-mediated cytotoxicity. In fact, the viability of cells transfected with surB and treated with TRAIL (1 or 2 ng/ml) was nearly equal to the viability of control cells treated with TRAIL (5 or 10 ng/ml), respectively.

Survivin downregulation by siRNA enhances TRAIL-induced apoptosis. To clarify whether surB transfection enhances TRAIL-induced apoptosis in Huh-7 cells, TUNEL assay was performed (Fig. 3). TUNEL-positive nuclei, small and bright fluorescence spots, were scattered in the cells either transfected with surB or treated with TRAIL (2 ng/ml), respectively. However, TUNEL-positive nuclei were apparently increased by combination of surB transfection with TRAIL treatment.

Discussion

We previously reported that IFN-α sensitized Huh-7 cells to TRAIL-induced apoptosis, in which IFN-α not only reduced survivin expression but also inhibited NF-κB activity and increased the expression of DR5, a death receptor of TRAIL (18). However, we could not address which was the key change directly relevant to the susceptibility to TRAIL, although ectopic expression of survivin partially rescued the IFN-α/TRAIL-induced apoptosis. In the present study, the downregulation of survivin by siRNA transfection sensitized Huh-7 cells to TRAIL-induced apoptosis as well as IFN-α, suggesting that downregulation of survivin is the major cause of IFN-α-mediated susceptibility of Huh-7 cells to TRAIL. Therefore, it is possible that the level of survivin is linked to the resistance against TRAIL-induced apoptosis in Huh-7 cells. This is consistent with previous observations in renal cell carcinoma cells and melanoma cells (16,17).

In this study, downregulation of survivin reduced the viability of Huh-7 cells through the induction of apoptosis. It was reported that an adenovirus expressing the dominant negative mutant of survivin caused spontaneous apoptosis in human cell lines of breast, cervical, prostate, lung and colorectal cancer but did not affect the viability of normal human cells, including fibroblasts, endothelium or smooth muscle cells (12,20). It was also reported that transduction of antisense oligonucleotides and siRNA against survivin induced apoptosis in HepG2 (21) and SMMC-7721 (22) human hepatoma cells, respectively. Therefore, it is likely that survivin supports the survival of hepatoma cells through inhibiting spontaneous apoptosis.

Survivin is abundantly expressed in hepatoma cells but not in normal hepatocytes (5,6), and TRAIL specifically induces apoptosis in cancer cells but not in normal hepatocytes (13,14). Therefore, the combination therapy of survivin knockdown and TRAIL administration may provide a new strategy for hepatoma treatment.

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DHMEQ, a novel NF-κB inhibitor, induces apoptosis and cell-cycle arrest in human hepatoma cells

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Abstract. Several reports have indicated that nuclear factor-κB (NF-κB) is constitutively activated in a variety of cancer cells including hepatoma cells and plays a key role in their growth and survival. Dehydroxymethylepoxyquinomicin (DHMEQ) derived from the structure of an antibiotic epoxyquinomicin C is a novel NF-κB inhibitor. In the present study, we evaluated the effect of DHMEQ on the NF-κB activity in human hepatoma cells, Huh-7, HepG2 and Hep3B, and the anti-tumor effect of DHMEQ on these cells *in vitro* and *in vivo*. DHMEQ inhibited the steady-state transcriptional activity of NF-κB in all hepatoma cells. DHMEQ blocked the constitutive DNA-binding activity and TNF-α-mediated nuclear translocation of NF-κB in Huh-7 cells. DHMEQ (5-20 μg/ml) dose-dependently reduced the viable cell number of all hepatoma cells. DHMEQ (20 μg/ml) induced apoptosis in all hepatoma cells, especially in Hep3B cells, and cell-cycle arrest in Huh-7 and HepG2 cells. These effects were accompanied by downregulation of proteins involved in anti-apoptosis (Bcl-xL, XIAP or c-IAP2) and cell-cycle progression (cyclin D1), and induction of proteins involved in pro-apoptosis (Bax) and cell-cycle retardation ($p21^{Waf1/Cip1}$), although the degree of changes by DHMEQ was different in each hepatoma cell type. Moreover, intraperitoneal administration of DHMEQ (8 mg/kg) significantly repressed the growth of Huh-7 tumor subcutaneously transplanted into BALB/c *nu/nu* athymic mice. Our results suggest that DHMEQ could qualify as a candidate for a new chemotherapeutic agent against human hepatoma.

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Abbreviations: ATL, adult T-cell leukemia; c-IAP, cellular inhibitor of apoptosis protein; DHMEQ, dehydroxymethylepoxyquinomicin; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; FLIP, FLICE inhibitory protein; HCC, hepatocellular carcinoma; IκB-α, inhibitor of κB-α; NF-κB, nuclear factor-κB; PBS-T, phosphate-buffered saline containing 0.1% Tween-20; PMSF, phenylmethylsulfonyl fluoride; PTTG, pituitary tumor-transforming gene; TNF-α, tumor necrosis factor α; TRAF, TNFR-associated factor; XIAP, X-chromosome-linked inhibitor of apoptosis protein

Key words: NF-κB, hepatoma, apoptosis, cell-cycle arrest

Introduction

Hepatocellular carcinoma (HCC), hepatoma, is one of the most common malignancies worldwide. It is estimated that half a million cases occur annually worldwide (1). Several strategies have been implemented for the treatment of patients with HCC, such as surgical resection, percutaneous ethanol injection, radiofrequency ablation, transcatheter arterial embolization, and liver transplantation. However, reduced liver function derived from underlying liver cirrhosis has a profound effect on all treatment decisions and interferes with the use of these therapeutic modalities for HCC except liver transplantations (2). In addition, multicentric tumor recurrence rates are very high, even in patients with HCC who receive curative treatment. Therefore, HCC is a tumor with high lethality (1,3), and a novel approach for the treatment of HCC is needed.

The nuclear factor kappa B (NF-κB), a transcription factor family, consists of several structurally-related proteins such as RelA (p65), RelB, c-Rel, p50/p105 and p52/p100, which form homo- or heterodimers with each other and regulate the expression of a number of genes (4,5). NF-κB plays a well-known function in the regulation of immune responses and inflammation, but growing evidence supports a major role in oncogenesis. NF-κB regulates the expression of genes involved in many processes that play a key role in the development and progression of cancer such as proliferation, migration and apoptosis (6-8). Aberrant or constitutive NF-κB activation has been detected in many human malignancies including HCC (6-9).

Dehydroxylmethylepoxyquinomicin (DHMEQ) is a novel NF-κB inhibitor produced by Dr Kazuo Umezawa (Keio

University, Japan), based on the structure of epoxyquinomicin C originally isolated from Amycolatopsis (10,11). DHMEQ has been reported to repress renal inflammation in rats and osteoclastogenesis in cultured bone marrow cells through inhibiting NF-κB activity (12,13). In addition, DHMEQ have shown anti-tumor activity against several cancer cells including prostatic cancer (14,15), thyroid cancer (16), malignant myeloma (17,18), breast cancer (19) and adult T-cell leukemia (ATL) cells (20,21), in which NF-κB is constitutively activated and contributes to the growth and survival of these cells. The present study was set up to determine the effects of DHMEQ in human hepatoma cells both *in vitro* and *in vivo* and to additionally elucidate the molecular mechanisms underlying the action of this agent.

Materials and methods

Cell culture and viability assay. The human hepatoma cell lines, Huh-7, HepG2 and Hep3B were maintained in RPMI containing 10% fetal bovine serum (FBS). DHMEQ was a generous gift from Professor Kazuo Umezawa (Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama, Japan). It was dissolved in DMSO to prepare a 10 μg/μl solution and subsequently diluted in culture medium to a final DMSO concentration of <0.2%. To analyze cell viability, 3×10^3 cells were placed into 96-well multiplates. One day later, the medium was replaced with fresh medium containing varying concentrations of DHMEQ or vehicle (0.2% DMSO) alone, and the cells were incubated for 48 h. Following removal of the medium and dead cells, viable cells were counted with Particle counter Z1 (Beckman Coulter, Inc., Fullerton, CA).

Reporter gene transfection assay. The pNFκB-luc (Stratagene, La Jolla, CA) containing four copies of the binding sequence of NFκB and firefly luciferase gene and pRL-CMV-luc (Promega, Madison, WI) containing the cytomegalovirus immediate early enhancer/promoter and expressing renilla luciferase gene were used in the assay. Cells were grown in 24-well multiplates in triplicate one day before transfection. In the next step, 1 μg of pNFκB-luc and 10 ng of pRL-CMV-luc were transfected into the cells using Lipofectin (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. After 6-h incubation, the medium was replaced with fresh medium containing varying concentrations of DHMEQ or vehicle (0.2% DMSO) alone, and the cells were incubated for 12 h. Luciferase activity in the cells was then determined by a dual-luciferase reporter assay system and a TD-20/20 luminometer (Promega).

Electrophoretic mobility shift assay (EMSA). Huh-7 cells were incubated with 20 μg/ml of DHMEQ for 1-24 h. The nuclear extract was prepared as described previously (22). As a positive control, the nuclear extract from HUT-78 cells containing NF-κB (23) was used. EMSA was performed using an EMSA kit (Panomics, Inc., Redwood City, CA) according to the manufacturer's protocol. Briefly, the same amount of protein from each nuclear extract was incubated with a biotin-tagged NF-κB probe; 5'-AGTTGAGGGGACTTTCCCAGGC-3' for 30 min at 15°C. The reaction mixture was electrophoresed using a 5% polyacrylamide gel containing 25 mM Tris-borate and 0.25 mM EDTA at 4°C and transferred to nylon membrane (Hybond-N+, Amersham Biosciences AB, Uppsala, Sweden). The blots were incubated with a detection agent in the kit and visualized with SuperSignal® West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL).

Immunofluorescent histochemistry. Huh-7 cells were seeded onto 11-mm glass coverslips in 24-well plates at 2×10^4 cells/well. The next day, the medium was replaced with serum-free medium, and the cells were pretreated with 20 μg/ml of DHMEQ or vehicle (0.2% DMSO) alone for 2 h then stimulated with 200 U/ml of TNF-α for 60 min. The cells were fixed with 4% paraformaldehyde in PBS for 10 min at 4°C, immersed in -20°C methanol for 10 min, and incubated in blocking buffer (5% normal horse serum in PBS) for 1 h. The cells were incubated with polyclonal rabbit anti-human p65 (RelA) (Biogenesis, Poole, UK) for 1 h at room temperature, washed three times in PBS, incubated with rhodamine-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h, washed in PBS, and mounted in Vectashield® Mounting Medium (Vector Laboratories Inc., Burlingame, CA). Immunofluorescence for rhodamine was analyzed by Olympus BX50 microscope (Tokyo, Japan) and images were digitally captured using a Nikon DXM 1200 digital camera (Tokyo, Japan).

Western blotting. The following antibodies were used in the experiments; rabbit polyclonal anti-human Bcl-xL, rabbit polyclonal anti-human XIAP, mouse monoclonal anti-human cyclin D1, mouse monoclonal anti-human $p21^{Waf1/Cip1}$ (Cell Signaling Technology, Inc. Beverly, MA), rabbit polyclonal anti-human c-IAP2, mouse monoclonal anti-human Bax (B-9), rabbit polyclonal anti-human PTTG (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), mouse monoclonal anti-human ß-actin (Sigma-Aldrich, Inc.). Cells were incubated with 20 μg/ml DHMEQ or vehicle (0.2% DMSO) alone for 24 h. Then cells were lysed by addition of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml of aprotinin, 1% NP-40, 0.5% sodium deoxycholate) for 10 min at 4°C, and insoluble material was removed by centrifugation at 14,000 rpm for 30 min at 4°C. The same amount of protein from each lysate (20 μg /well) was subjected to 15% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes which were then blocked for 1.5 h using 5% non-fat dried milk in PBS containing 0.1% Tween-20 (PBS-T), washed with PBS-T and incubated at 4°C overnight in the presence of each primary antibody. The membranes were washed with PBS-T and incubated with sheep anti-mouse IgG or donkey anti-rabbit IgG coupled with horseradish peroxidase (Amersham Biosciences AB). The enhanced chemiluminescence system (SuperSignal® West Pico Chemiluminescent Substrate; Pierce Chemical Co.) was used for detection.

Determination of apoptosis and cell-cycle modulation. Cells (4×10^5) were plated in 100 mm dish, incubated overnight, and treated with 20 μg/ml of DHMEQ or vehicle (0.2% DMSO) alone for 24 h. Cells were washed in PBS and permeabilized with 70% ethanol at 4°C at least overnight. After centrifugation,