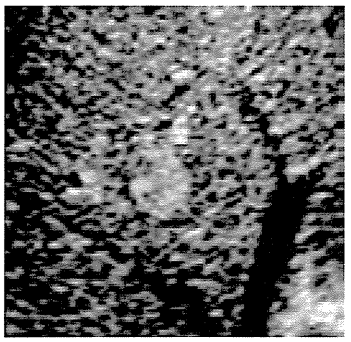
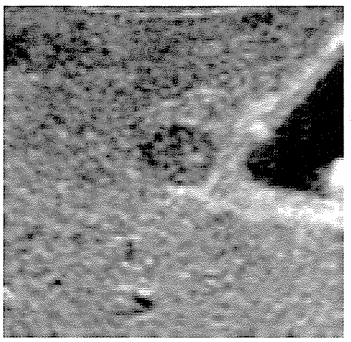
参考図

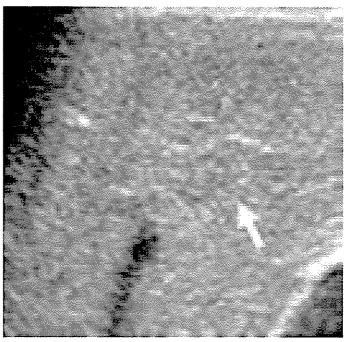
肝細胞癌



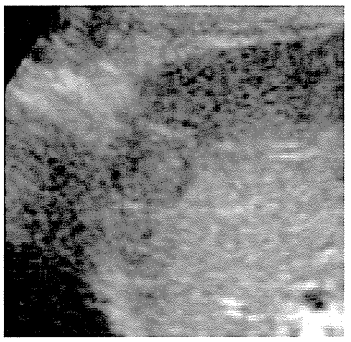
高エコー（結節型）



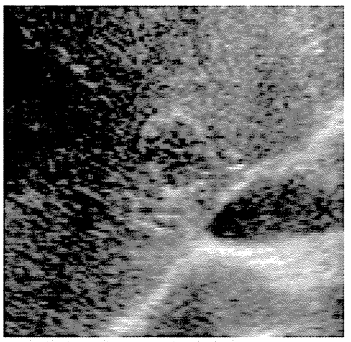
低エコー（結節型）



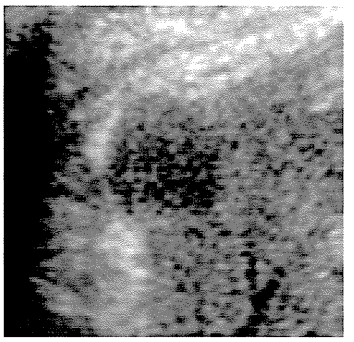
等エコー（結節型）



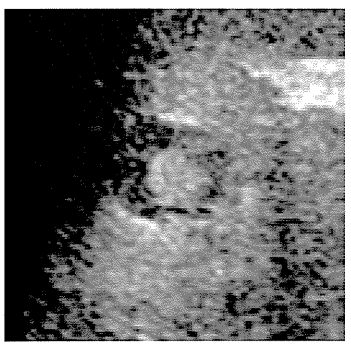
辺縁低エコー（結節型）



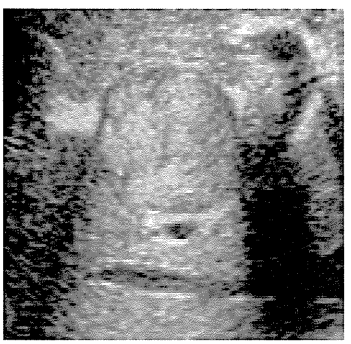
bright loop（結節型）



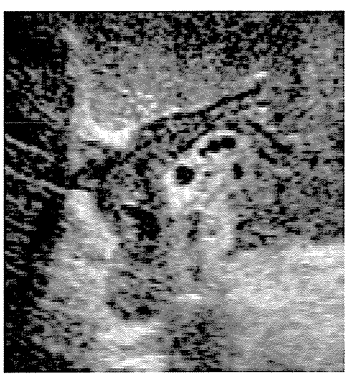
hump sign（結節型）



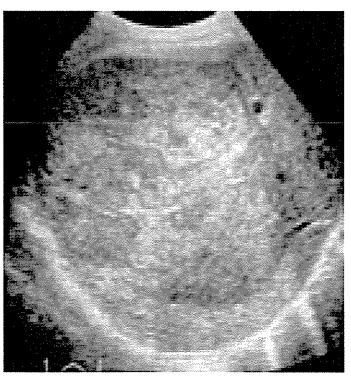
nodule in nodule



mosaic pattern



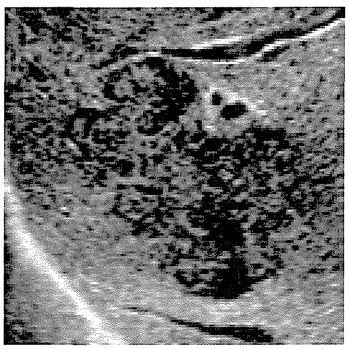
門脈腫瘍塞栓



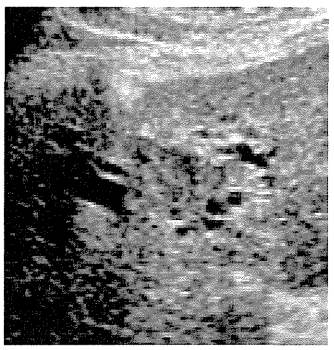
塊状型

参考図

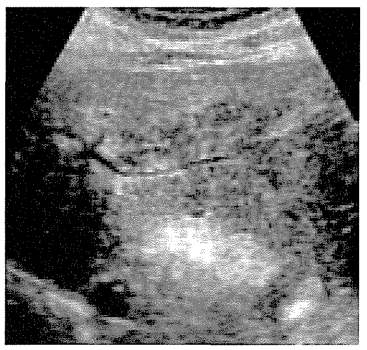
肝内胆管癌（胆管細胞癌）



境界不明瞭結節

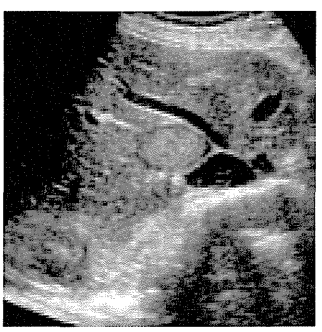


末梢胆管の拡張

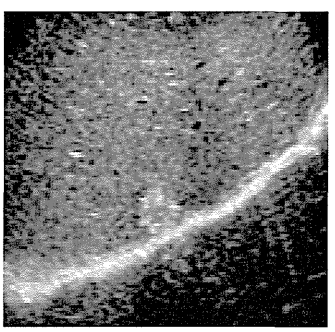


腫瘍を貫く血管

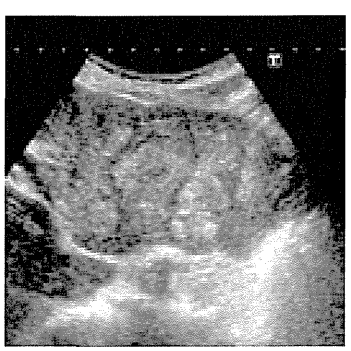
転移性肝腫瘍



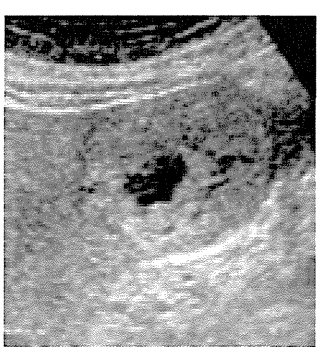
bull's eye pattern



高エコー



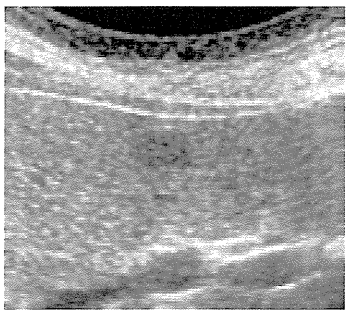
cluster sign



中心部に無エコー域

参考図

肝細胞腺腫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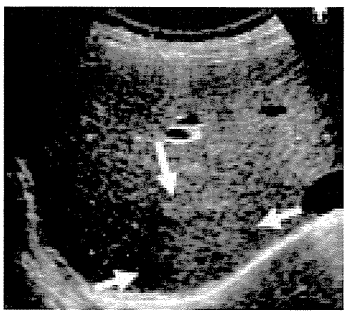
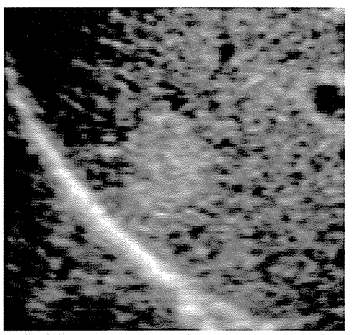

等エコー

肝血管腫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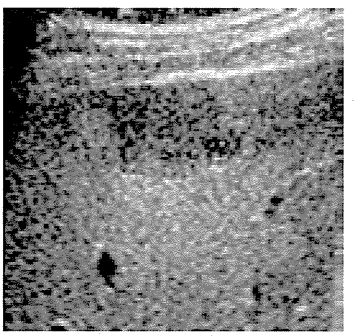
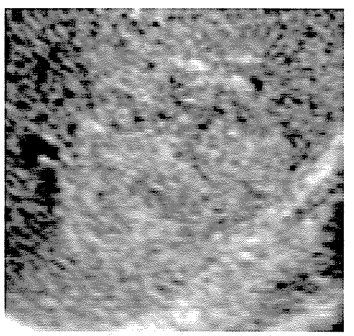
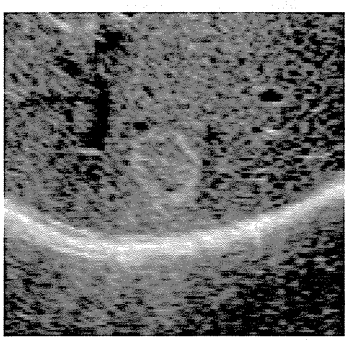
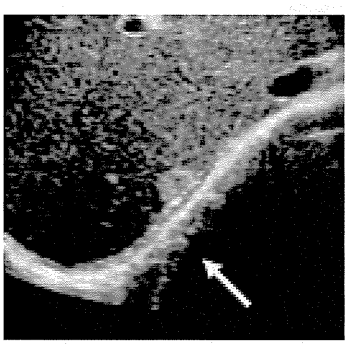
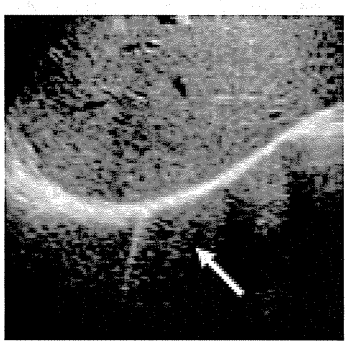

低エコー

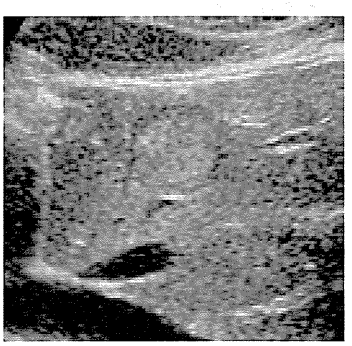

混合エコー

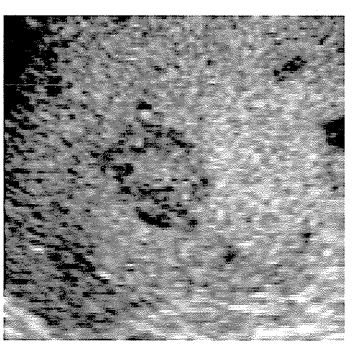

辺縁高エコー帯




chameleon sign

限局性結節性過形成（FNH）


高エコー


中心高エコー

いため腫瘍としたが，主に胃癌や大腸癌などの消化器系の癌の典型像を示す．

注 2）腫瘍の大きさは質的診断において間接所見であるが，腫瘍の内部構造とは密接な関係があると考えられるので肝細胞癌の結節型においてのみサイズ別に代表する所見を記載した．

注 3）随伴所見や特徴的な形態変化は間接所見であるが，質的診断をするうえで重要な情報となりうるので付加所見として記載した．

注 4）肝細胞癌の肉眼分類として小結節境界不明瞭型，浸潤型，びまん型があるが，これらは腫瘤を形成せず，エコーレベルも肝実質との差が少なく存在が認識しにくいので診断基準からは除いた．しかし，びまん型や浸潤型は門脈や肝静脈の腫瘍栓を有する場合があり，この所見によって診断されることがある．小結節境界不明瞭型は組織学的には早期肝細胞癌に相当する．CT もしくは MRI などの他の画像診断法の併用が必要となる．また，単純結節型，単純結節周囲増殖型，多結節癒合型は結節型として所見を記載した．

注 5）肝辺縁に存在する肝細胞癌では腫瘤の一部が肝表面より突出する所見（hump sign）が認められることがある．

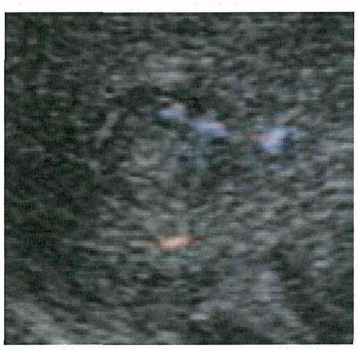
注 6）異型結節は基本的には肝細胞癌結節型（2 cm 以下）の所見に類似し鑑別は困難である．

注 7）肝内胆管癌（胆管細胞癌）には腫瘤形成型，胆管浸潤型，肝内胆管発育型があるが，ここで記載した所見は腫瘤形成型の所見である．

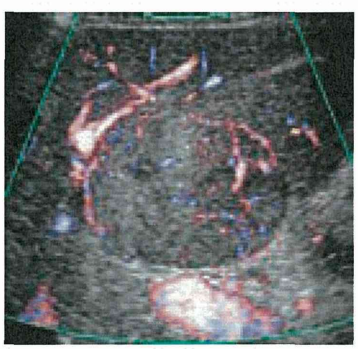
5.2 ドプラ所見

ドプラ所見は，**Table 2** に示すように腫瘤内の血流の多寡，血管の走行，血流性状（拍動波，定常波），付加所見などと[5, 6]，B モード所見と合わせて鑑別診断を行う．血流の方向についても評価することが望ましい．

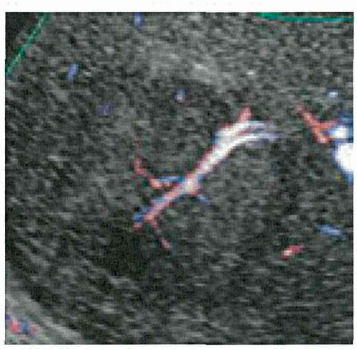
参考図



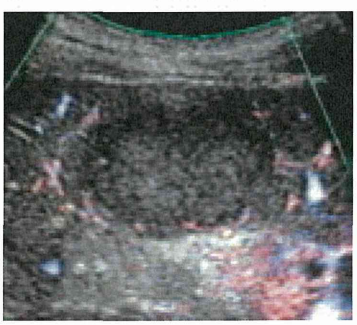
肝細胞癌（2 cm 以下）



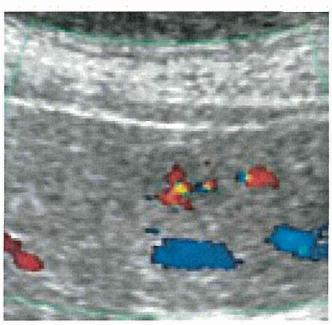
肝細胞癌（バスケットパターン）



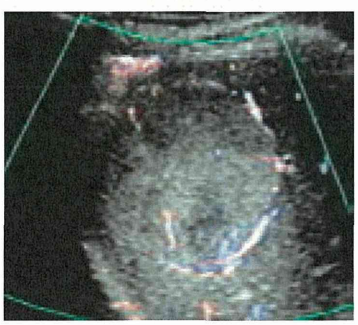
肝内胆管癌



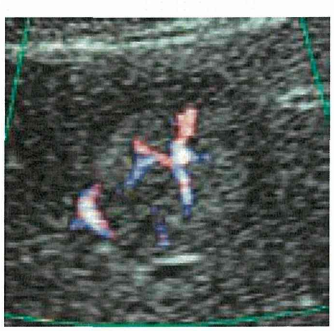
転移性肝腫瘍



肝細胞腺腫



肝血管腫



FNH

Table 2 ドプラ所見

主分類	細分類	血流の多寡	血管の走行	血流性状	付加所見
肝細胞癌	結節型（2 cm以下）	少ない	時に腫瘍内部および周辺に線状もしくは点状	定常性 時に拍動性	血流信号が認められないことが多い
	結節型（2 cmを越える）	多い	バスケットパターン（周辺から中心に向かう）	拍動性 時に定常性	A-P shuntや腫瘍塞栓を認めることもある
	塊状型	多い	不整な血管，バスケットパターン	拍動性	門脈内に拍動流を認める場合腫瘍塞栓やA-P shuntをの存在を疑う．
肝内胆管癌（胆管細胞癌）		少ない	腫瘍周辺に圧排 腫瘍内に既存血管の残存	拍動性 定常性	腫瘍周辺の一部のみ血流信号を認めることが多いが，内部でも見られる場合がある．
転移性肝腫瘍		少ない	腫瘍周辺に 圧排 腫瘍内に既存血管の残存	拍動性 定常性	腫瘍周辺部に血流信号を認めることが多いが，中心部はあまり認めない．原発巣によっては血流が多いことがある．
肝細胞腺腫		多い	腫瘍境界から取り囲むように内部に細い血管が流入	拍動性 時に定常性	
肝血管腫		少ない	腫瘍辺縁部に点状	定常性 時に拍動性	A-P shuntを認めることもある．血流が豊富な場合がある．
限局性結節性過形成（FNH）		多い	腫瘍中心部から流入し辺縁に広がる spoke-wheel pattern	拍動性	

注1）いずれも典型的な所見を示した．転移性肝腫瘍（癌）は上皮性，非上皮性を区別していないため腫瘍としたが，主に胃癌や大腸癌などの消化器系の癌の典型像を示す．

注2）肝細胞癌は腫瘍の大きさやパターンにより特有の血流パターンを示すためBモード所見の細分類を用いた．血流の方向を加味して解釈するのが望ましい．一部の肝細胞癌結節型（2 cm以下）は流入する定常性血流のみを認めることが多く，基本的には異型結節との鑑別は困難である．

注3）肝内胆管癌（胆管細胞癌）には腫瘤形成型，胆管浸潤型，肝内胆管発育型があるが，ここで記載した所見は腫瘤形成型のドプラ所見である．

5.3 造影所見（時相，イメージの定義）

肝臓は肝動脈（25～30%）と門脈（70～75%）の2重の血行支配であり，超音波造影剤を静脈から投与すると3つのオーバーラップする時相（phase，造影超音波検査における造影剤投与後の経時的撮像タイミング）が観察される．時相に関しては以下の如く定義する．

血管相（vascular phase，造影超音波検査において造影剤が血管内に存在している時相）と後血管相（post vascular phase，血管内の造影剤濃度が十分に低下し，造影剤による血管の造影効果が失われた時相）に分類し，血管相はさらに，動脈優位相（arterial［predominant］phase，臓器実質および腫瘍が動脈由来の造影剤により造影される時相）と門脈優位相（portal［predominant］phase，肝内門脈枝が造影された後肝実質が造影される時相）に分ける．動脈優位相では腫瘤内の血管構築像，腫瘤の灌流像が得られる．門脈優位相では腫瘤の造影剤のwashoutと肝実質相の染まりの輝度を比較する．動脈優位相で得られる画像を血管イメージ（vascular image）および灌流イメージ（perfusion image），後血管相で得られる画像を後血管イメージ（post vascular image）と呼ぶ．各疾患の造影所見を**Table 3**に示す．

注1）血管相は質的診断を，後血管相は存在診断を主目的として使用される．

注2）後血管イメージは，「クッパーイメージ（Kupffer image）」とも呼ばれるが，この点に関しては異論もあり今後の検討が必要である[7-10]．

注3）1つの目安であるが，動脈（優位）相は造影剤静脈内投与後約30秒まで，門脈（優位）相はそれ以後から約120秒まで，後血管相は約10分以降とされる．ただし，肝機能もしくは腫瘤の状態により個人差のあることには留意する[11]．

注4）いずれも典型的な所見を示した．転移性肝腫瘍は上皮性，非上皮性を区別していないため腫瘍としたが，主に胃癌や大腸癌などの消化器系の癌典型像を示す．

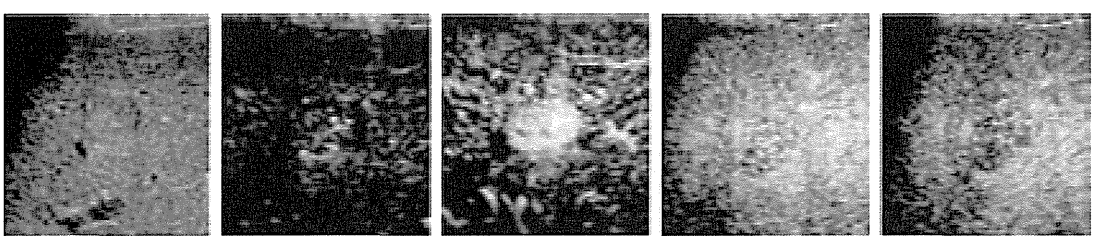
注5）後血管相の撮像時に血管相では気付かれなかった新たな病変が発見された場合は再度造影剤を注入してその結節の血管相を評価することが可能であ

Table 3 造影超音波による質的診断

主分類	細分類	血管相（vascular phase）		後血管相（post vascular phase）	付加所見
		動脈（優位）相（arterial [predominant] phase）	門脈（優位）相（portal [predominant] phase）		
肝細胞癌	結節型（2 cm以下）	造影剤が流入する場合もあるが血管として描出される本数は少ない	肝実質と同程度もしくは低下して造影される	肝実質に比して軽度低下もしくは低下	動脈（優位）相で濃染しない症例もある
	結節型（2 cmを越える）	バスケットパターン，血管増生，不整な流入血管 肝実質に比し強い濃染	肝実質に比し低下して造影される 非造影部位の存在	欠損もしくは不完全な欠損	後血管相で点状のシグナルが残存することあり
	塊状型	バスケットパターン，血管増生，不整な流入血管 肝実質に比し強く不均一な濃染	肝実質と低下して造影される 非造影部位が存在	欠損もしくは不完全な欠損 腫瘍の輪郭は不整	染影される腫瘍塞栓の描出されることあり
肝内胆管癌（胆管細胞癌）		辺縁に血管影 辺縁のリング状濃染	腫瘍辺縁のリング状濃染 肝実質に比して低下して造影される	明瞭な欠損もしくは不完全な欠損	中央を突き抜ける線状の血管を認めることもある 全く染影されない場合もあり
転移性肝腫瘍		腫瘍内の点状の血管影，辺縁のリング状濃染	腫瘍辺縁のリング状濃染 肝実質に比して低下して造影される	明瞭な欠損 腫瘍の輪郭は不正	血管増生のある転移性肝腫瘍は動脈（優位）相の所見は肝細胞癌に類似する
肝細胞腺腫		境界から中央に向かって細かな血管が流入する，血管増生，肝実質に比し軽度の濃染	肝実質に比し造影される	同等もしくは不完全な欠損	出血・壊死を伴う場合は非造影部位を生じる
肝血管腫		辺縁から中央に向かって濃染され始める．辺縁が点状もしくは斑状に濃染される	辺縁が斑状に濃染される．中央へ濃染が進み，中心部は造影されないことが多い．	肝実質と同等，一部造影されない場合有り（血栓，線維化など）	小さなものでは急速に中央に向かって濃染される場合もある．
限局性結節性過形成（FNH）		spoke-wheel pattern，中央から外側に向かって極めて短時間に肝実質より濃染	肝実質より濃染 造影の低下する部分もある（中心瘢痕）	造影は肝実質と同等，造影の低下する部分もある（中心瘢痕）	

参考図

肝細胞癌結節型（2 cm以下）



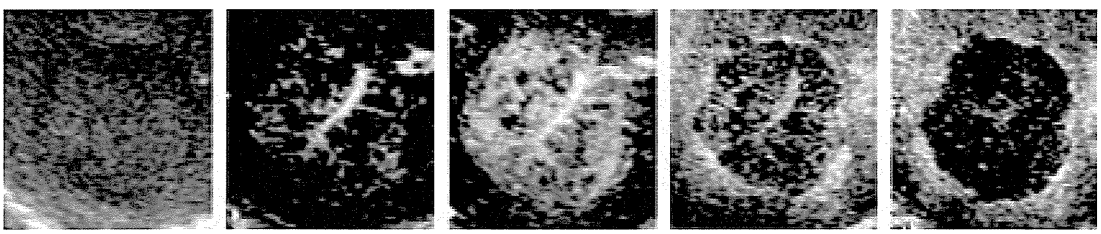
造影前　動脈優位相(血管像)　動脈優位相(灌流像)　門脈優位相　後血管相

参考図

肝細胞癌結節型（2 cm を超える）

造影前　動脈優位相（血管像）　動脈優位相（灌流像）　門脈優位相　後血管相

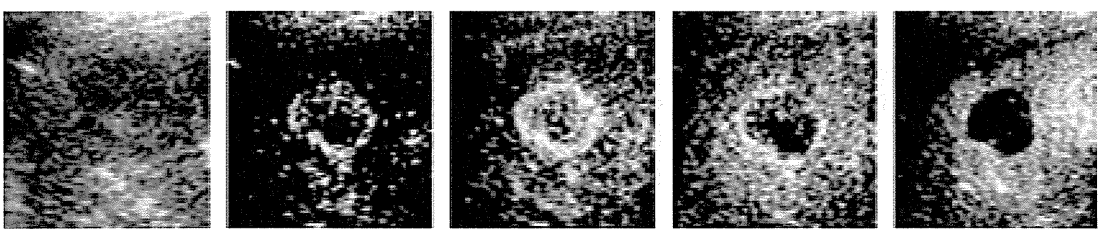
肝内胆管癌（胆管細胞癌）



造影前　動脈優位相（血管像）　動脈優位相（灌流像）　門脈優位相　後血管相

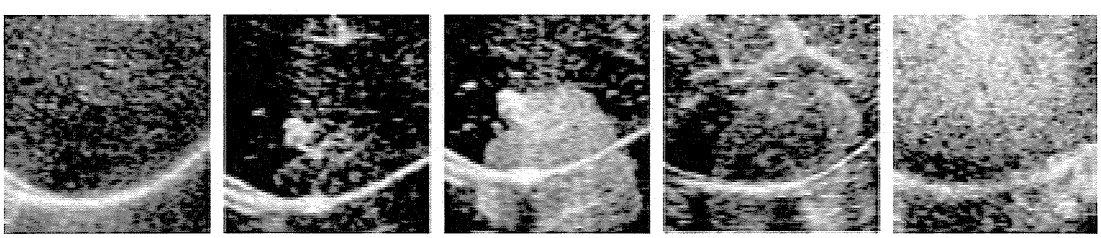
参考図

転移性肝腫瘍（胃癌）



造影前　動脈優位相（血管像）　動脈優位相（灌流像）　門脈優位相　後血管相

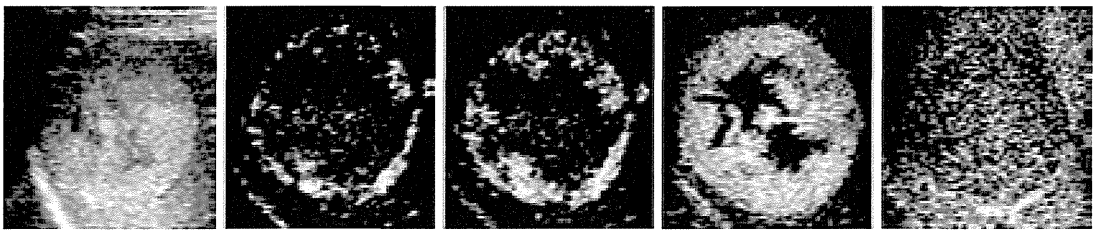
肝細胞腺腫



造影前　動脈優位相（血管像）　動脈優位相（灌流像）　門脈優位相　後血管相

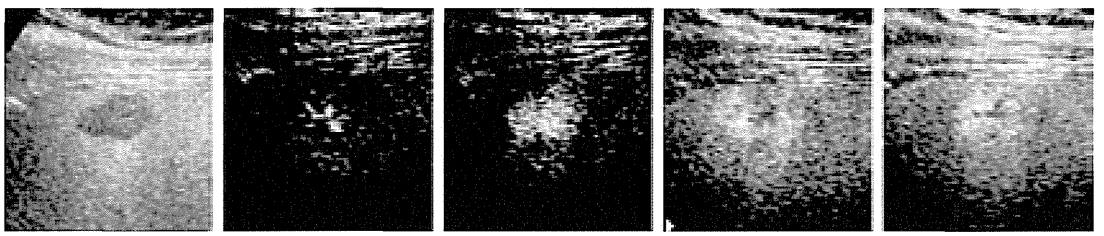
参考図

肝血管腫



造影前　動脈優位相(血管像)　動脈優位相(灌流像)　門脈優位相　後血管相

限局性結節性過形成（FNH）



造影前　動脈優位相(血管像)　動脈優位相(灌流像)　門脈優位相　後血管相

る（defect reperfusion image）[12].

注6）血管相の門脈（優位）相では一時高MIとして腫瘤内のbubbleを破壊して観察すると，腫瘤内の血流動態が再度観察可能となる（replenishment method）．ただし，動脈のみの血流ではない．

注7）異型結節と肝細胞癌の鑑別においては他の画像を含めた総合評価で行うことが望ましい．

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特集II B型肝炎の抗ウイルス療法の進歩と耐性

B型肝炎に対する核酸アナログ投与例の長期予後*

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Key Words: hepatitis B, nucleos(t)ide analogue, carcinogenesis, hepatitis B virus core-related antigen, hepatocellular carcinoma

はじめに

B型肝炎は全世界において3億5000万人の持続感染者の存在が推測されており，年間60～100万人がB型肝炎に起因する慢性肝炎，肝硬変，肝癌で死亡していると推測されている[1]．近年，B型肝炎に対する治療薬である核酸アナログ製剤が登場し，それ以前に使用されることが多かったインターフェロンと比較して副作用が少なく，抗ウイルス効果が高いため，臨床の現場で広く使用されるようになり，B型肝炎の治療環境は大きく変化してきている．加えて免疫抑制剤・化学療法により発症するB型肝炎(HBVの再活性化)が注目されるようになり[2]，核酸アナログ投与例はさらに増加してきている．

今回われわれは，当院で経験したB型肝炎患者における核酸アナログ投与と肝発癌に関する検討を行ったので若干の考察を含め報告する．

対象と方法

1998～2008年の10年間に当院で経験したB型肝炎患者1,973例中，①HBs抗原が6か月以上陽性，②経過観察開始から3年以上経過，③alanine aminotransferase(ALT)を年2回以上測定，④発癌例では経過観察開始後1年以降で発癌，⑤核酸アナログ服用例では1年以上服用，のすべてを満たしたのは785例であった．これら785例中，核酸アナログ投与例は148例で，非投与例は637例であった．さらにpropensity score matching法を用いて経過観察開始時の背景因子(年齢，性別，ALT，血小板，HBV-DNA量，HBe抗原，Child-Pugh分類)をマッチさせ，核酸アナログ投与群および非投与群をそれぞれ117例ずつ選択し，計234例を今回の対象とした(図1)．

核酸アナログ投与群および非投与群の患者背景を表1に示す．背景因子として，治療開始時もしくは経過観察開始時の年齢，性別，ジェノタイプ(A/B/C)，HBV-DNA量，HBe抗原，プレコアと基本コアプロモーター(BCP)の野生型/変異型，血小板，ALTに有意差は認められなかった．

核酸アナログ製剤の投与状況は，ラミブジンが18例，ラミブジン＋アデフォビルが28例，エンテカビルが71例(エンテカビル開始が30例，ラミブジンからエンテカビルに変更が41例)であった．また，観察期間中央値は7.1年(1.5～18.3)であった．

* Long-term outcome of hepatitis B under treatment with nucleos(t)ide analogues.

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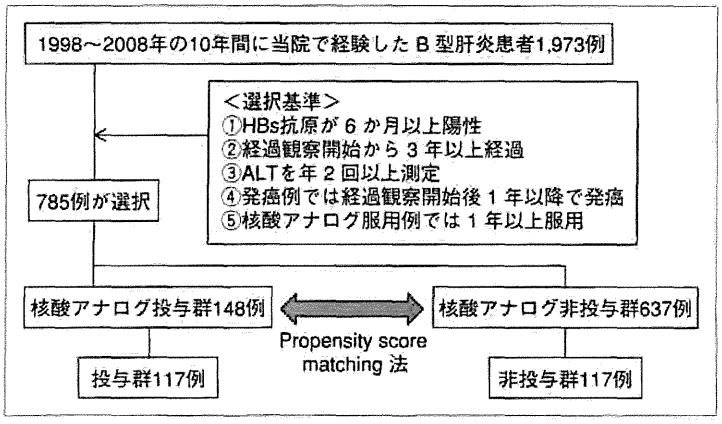

図1 対象患者の選択

表1 患者背景

	投与群(n=117)	非投与群(n=117)	P値
年齢(歳)	52(27～77)	52(21～77)	0.922
性別(女性/男性)	44/73	45/72	0.893
ジェノタイプ(A/B/C)	1/4/109	4/7/85	0.123
HBV-DNA(log copies/ml)	6.7(2.6～9.6)	6.5(2.1～9.6)	0.084
HBeAg(+/−)	57/60	58/59	0.896
プレコア(野生型/変異型)	22/87	16/75	0.640
BCP(野生型/変異型)	22/88	17/70	0.936
血小板($\times 10^4$/m^3)	14.3(3.2～26.2)	14.6(3.7～39.6)	0.634
ALT(IU/ml)	68(7～1,088)	55(9～3,410)	0.098

検討内容は血小板，ALT, gamma glutamyl transpeptidase(γ-GTP)，total bilirubin(T. bil)，alkaline phosphatase(ALP)，albumin(Alb)，alpha-fetoprotein(AFP)，HBV-DNA量の各項目に対して，治療開始時もしくは経過観察開始時および経過観察開始後の時間軸を考慮に入れた積分平均値[3]を算出し，核酸アナログ投与群と非投与群の比較を行った．なお，発癌例の血液データは発癌1年前までの値を使用した．さらに，発癌率および多変量解析を用い発癌に関与する因子につき検討を行った．また，HBコア関連抗原(HBcrAg)の測定が可能であった一部の症例では，HBcrAgと発癌との関連についても検討を行った．

統計ソフトはSPSS(ver 18)を用いて行い，2群間の比較はMann-WhitneyのU検定，発癌率の検定はKaplan-Meier法，多変量解析にはCox比例ハザードモデル(変数増加法)をそれぞれ使用し，$P<0.05$を有意差ありと判定した．

結　果

経過観察開始後の血液・生化学データ(積分平均値)を表2に示す．核酸アナログ投与群は非投与群と比較して，血小板が高値($P=0.006$)，ALTが低値($P<0.001$)，γ-GTPが低値($P=0.043$)，ALPが低値(0.013)，Albが高値($P<0.001$)，AFPが低値($P<0.001$)となり肝機能は明らかに改善が認められた．HBV-DNA量(積分平均値)に関しても同様に核酸アナログ投与群は2.5log copies/ml(1.2～8.9)，非投与群は4.6log copies/ml(2.1～9.3)で，投与群において有意な低下が認められた($P<0.001$)(図2)．

続いて，治療開始時もしくは経過観察開始時の年齢(40歳以下/超)，性別，核酸アナログ投与の有無，プレコア，BCP, HBV-DNA量(5.0log copies/ml以下/超)，HBcrAg(3.0log U/ml以下/超)，血小板(15×10^4/m^{3}以下/超)，ALT(40IU/l以下/超)，γ-GTP(56IU/l以下/超)，ALP(338IU/l以下/

表2　経過観察開始後の血液・生化学データ(積分平均値)

	投与群(n=117)	非投与群(n=117)	P値
血小板(×10⁴/m³)	17.0(3.3～37.2)	14.8(3.3～29.6)	0.006
ALT(IU/l)	28.2(8.5～88.9)	39.1(12.2～737.5)	<0.001
γ-GTP(IU/l)	27.0(10.9～267.6)	36.2(9.5～269.7)	0.043
T.Bil(mg/dl)	0.7(0.3～2.0)	0.7(0.3～2.6)	0.155
ALP(IU/l)	242.7(113.5～1028.8)	265.2(140.0～1247.6)	0.013
Alb(g/dl)	4.4(3.0～5.0)	4.0(2.4～4.8)	<0.001
AFP(ng/ml)	2.15(0.8～106.0)	4.50(0.9～723.8)	<0.001

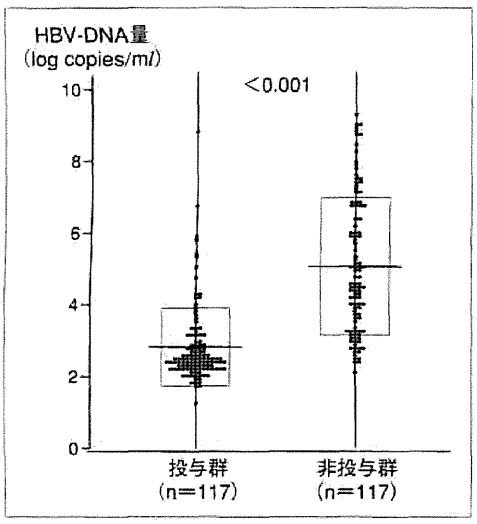

図2　核酸アナログ投与の有無における経過観察中のHBV-DNA量の比較

超)を投入因子として発癌に関与する因子について検討したところ，年齢(40歳超：ハザード比4.424, 95%信頼区間1.346～14.542, P=0.014)，核酸アナログ投与の有無(投与なし：ハザード比4.039, 95%信頼区間1.662～9.820, P=0.002)，BCP(変異型：ハザード比11.952, 95%信頼区間1.630～87.649, P=0.015)，HBcrAg(3.0logU/ml超：ハザード比4.334, 95%信頼区間1.313～14.309, P=0.016)，γ-GTP(56IU/l超：ハザード比2.539, 95%信頼区間1.347～4.784, P=0.004)が発癌に関与する因子として選択された(表3)．

さらに，核酸アナログ投与の有無別に累積発癌率を検討したところ，5年/10年の発癌率はそれぞれ投与群で5.2%/8.4%，非投与群で8.7%/36.8%となり，非投与群で有意に発癌率が高かった(ハザード比2.838, 95%信頼区間1.243～6.478, P=0.013)(図3)．BCP別の累積発癌率の検討では，5年/10年/15年の発癌率はそれぞれ野生型群(n=39)で0.0%/0.0%/12.5%，変異型群(n=158)で8.4%/41.3%/52.5%となり，変異型群で有意に発癌率が高かった(ハザード比12.360, 95%信頼区間1.699～89.944, P=0.013)(図4)．また，HBcrAg(3.0logU/ml以下/超)別の累積発癌率の検討では，5年/10年/15年の発癌率はそれぞれ3.0logU/ml以下群(n=46)で0.0%/19.1%/39.3%，3.0logU/ml超群(n=171)群で8.4%/35.1%/49.2%となり，3.0logU/ml超群で発癌率が高くなる傾向を認めた(ハザード比2.095, 95%

表3　肝細胞癌の発癌に関する因子(Cox比例ハザードモデル)

		ハザード比(95%信頼区間)	P値
年齢(歳)	≦40	1	0.014
	>40	4.424(1.346～14.542)	
核酸アナログ投与	投与あり	1	0.002
	投与なし	4.039(1.662～9.820)	
BCP(野生型/変異型)	野生型	1	0.015
	変異型	11.952(1.630～87.649)	
HBcrAg(logU/ml)	≦3.0	1	0.016
	>3.0	4.334(1.313～14.309)	
γ-GTP (IU/l)	≦56	1	0.004
	>56	2.539(1.347～4.784)	

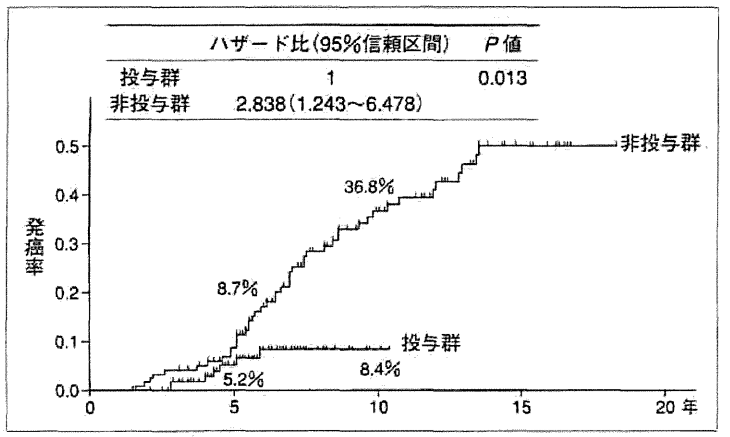

図3 核酸アナログ投与の有無による肝細胞癌の発癌率

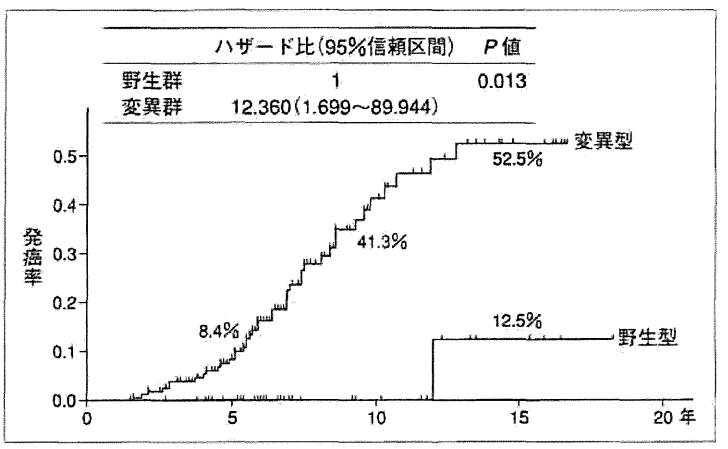

図4 BCP別の肝細胞癌の発癌率

信頼区間0.976～4.450, $P=0.0579$)(図5).

考 察

B型肝炎の発癌に関与する因子に関してはこれまで数多くの報告がなされている. Chenら[4]は, HBe抗原陰性, HBV-DNA<4 log copies/mlで肝硬変, 肝癌, ALT高値が認められない1,932人の患者をHBV非活動性キャリア群とし, それに対するコントロール群(非B非C)18,137人との平均観察期間13.1年の経過を報告している. 報告によると発癌率はHBV非活動性キャリア群が0.06%/年, コントロール群が0.04%/年であり, さらに肝関連死に関しては両群とも0.02%/年であった. 多変量解析ではHBV非活動性キャリアがコントロールに比較して, 発癌に関してはハザード比4.6(95%信頼区間2.5～8.3), 肝関連死に関してはハザード比2.1(95%信頼区間1.1～4.1)であり, 加えて高齢や飲酒も独立した発癌の危険因子であると結論している.

B型肝炎の背景肝の違いによる年間発癌率も報告がされており[5], 非活動性キャリアは0.2%未満, 慢性肝炎は1%未満, 代償性肝硬変は2～3%そして非代償性肝硬変は7～8%とされ, 慢性肝障害の進行とともに発癌率の上昇が認められている. また, 宿主側の因子では肝硬変, 糖尿病, 肥満, 飲酒, 高齢, 男性, 家族歴, 人種(アジア人, アフリカ人)があげられ, ウイルス側の因子ではHBV-DNA量, HBe抗原陽性, ジェ

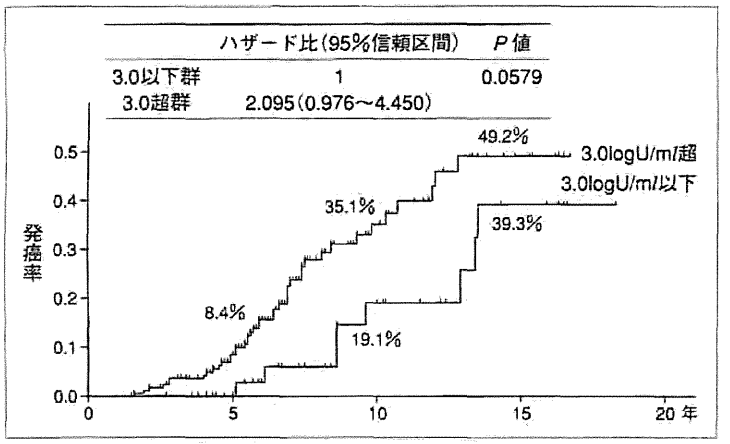

図5　HBcrAg値(3.0logU/ml)別の肝細胞癌の発癌率

ノタイプ，プレコアやBCP変異，HCVやHIVとの重複感染等が発癌に関与していると報告されている[6]．

今回のわれわれの検討では，多変量解析によって年齢(40歳超)，核酸アナログ投与なし，BCP(変異型)，HBcrAg(3.0logU/ml超)，γ-GTP(56IU/l超)が発癌に関与する因子として選択された．年齢やBCPに関してはこれまで報告と一致しており，γ-GTPに関しても詳細な検討はできていないが，飲酒が関係しているものと考えられる．

核酸アナログ製剤と肝発癌に関してはLiawら[7]が大規模なランダム化比較試験(RCT)(the Cirrhosis Asian Lamivudine Multicentre Study)にてラミブジンによる肝発癌抑制効果と肝関連死の低減効果を証明している．また，本邦においてはMatsumotoら[8]がラミブジンによる肝発癌抑制効果を報告している．今回のわれわれの検討例では核酸アナログ投与例はラミブジンのみならず，ラミブジン＋アデフォビル，エンテカビル(ラミブジンからの変更例も含む)と多岐にわたるが，投与例は肝発癌の抑制が認められたとともに，非投与群と比較してHBV-DNA量の低下が認められ，さらに血小板やAlbが高値となり，ALT等が低値となる結果が得られ，肝機能の維持・改善効果が認められた．このような効果はたとえ核酸アナログ投与例で発癌が認められた場合でも，治療上，非常に有用であると考えられた．

今回の検討ではHBcrAg高値例も発癌に関与する因子として選択された．HBcrAgは肝組織中のcovalently closed circular DNA(cccDNA)量を反映していると考えられている[9]．Hosakaら[10]は核酸アナログ投与例において，肝細胞癌の再発はHBcrAg量が多い症例において有意に高率であったと報告している．今回の検討では経過観察開始時の値での検討であり，核酸アナログ開始後のHBcrAg量の変化と発癌の関係など，今後さらなる検討が必要であると考えられた．

おわりに

今回の検討では，B型肝炎患者に核酸アナログを投与することによりHBV-DNA量の低下および肝機能の維持・改善が認められるとともに，発癌が抑制されることが明らかとなった．年齢や肝機能，BCP変異，さらにはHBcrAg量なども検討し，必要であれば，タイミングを逃すことなく積極的に核酸アナログを投与することが重要であると考えられた．

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Novel method to measure serum levels of des-gamma-carboxy prothrombin for hepatocellular carcinoma in patients taking warfarin: A preliminary report

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Des-gamma-carboxy prothrombin (DCP) is a useful tumor marker for hepatocellular carcinoma (HCC), but its utility is limited in patients taking vitamin K antagonists. We evaluated the NX-DCP ratio, a newly developed method to measure serum DCP, for its ability to identify DCP elevation induced by HCC in this patient subpopulation. Conventional DCP measurements and the NX-DCP ratio were compared in patients with and without HCC, all of whom were taking the vitamin K antagonist warfarin. We found no differences in conventional DCP measurements between patients with and without HCC due to warfarin treatment. In contrast, the NX-DCP ratio was significantly higher in patients with HCC; the NX-DCP ratio in all patients without HCC was <1.50. When the cut-off was fixed at 1.50, sensitivity and specificity for HCC diagnosis were 60.0% and 100.0%, respectively, which are comparable to those of conventional DCP measurements in patients not taking warfarin. The novel NX-DCP ratio identifies patients on warfarin with elevated DCP due to HCC and is useful as a tumor marker for HCC in this patient subpopulation. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2012.02232.x, 2012)

Prothrombin, or coagulation factor II, is a 71 600 Da protein that consists of three regions: fragment 1; fragment 2; and prothrombin. Fragment 1 consists of 156 amino acids, including 41 amino acids forming an *N*-terminal gamma-glutamic acid (Gla)-containing domain. Prothrombin is first synthesized in the liver as a precursor with 10 glutamic acid (Glu) residues, which are then modified to Gla residues by gamma-glutamylcarboxylase in the presence of vitamin K, O_2, and CO_2 before it is released into the bloodstream.

However, in the absence of vitamin K or in the presence of vitamin K antagonists, gamma-carboxylation is impaired, and prothrombin with the remaining Glu residues, which is inactive with respect to coagulation, is released into the bloodstream.[1] Prothrombin in this form is called des-gamma-carboxy prothrombin (DCP) or protein induced by vitamin K absence/antagonist-II (PIVKA-II). As the number of Glu residues unconverted to Gla varies, DCP is present as a mixture of prothrombin with various numbers of Glu residues, ranging from 1 to 10. In addition, because the Gla residue can bind to calcium, it is known that the 3-D protein structure of DCP will be different in the presence of calcium, and depends on the number of Glu residues.[2]

Des-gamma-carboxy prothrombin is frequently found in the blood of patients with hepatocellular carcinoma (HCC). Because DCP is elevated in many patients with HCC but not in patients with chronic hepatitis or cirrhosis without HCC,[3] it has been routinely used as a tumor marker of HCC in clinical settings.[4–6] However, serum DCP levels are also increased in the absence of HCC when there is a shortage of vitamin K or in the presence of vitamin K antagonists.[7] The value of DCP as a marker of HCC, therefore, is significantly reduced in patients who are taking vitamin K antagonists such as warfarin.

Previous studies reported differences in the number of Glu residues in DCP between patients with HCC and patients taking vitamin K antagonists.[8,9] Conventionally, DCP is measured using a mAb produced by the cell line MU-3 (Picolumi PIVKA-II; EIDIA, Tokyo, Japan), which reportedly reacts predominantly with DCP with 9–10 Glu residues. MU-3 had lower affinity for DCP with one to five Glu residues.[10] However, measuring DCP with this antibody alone can not differentiate between HCC-induced and vitamin K antagonist-associated elevations of DCP, making it difficult to evaluate whether rises in DCP are caused by HCC in patients taking vitamin K antagonists.

In the present study, we attempted to identify HCC-induced DCP in patients with HCC taking the vitamin K antagonist warfarin through the use of two mAbs against DCP, P-11 and P-16 (Sekisui Medical, Tokyo, Japan), which have a reactivity profile different from MU-3. We found clinical utility in DCP as a marker for HCC in patients taking warfarin when measured with the combination of MU-3, P-11, and P-16.

Materials and Methods

Preparation of electrochemiluminescence immunoassay (ECLIA) reagents with P-11 and P-16. Magnetic beads coated with P-16 mAb (Sekisui Medical) were prepared as follows: 1 mL of 30 mg/mL magnetic bead suspension (Dynabeads M-450 Epoxy; Life Technologies, Carlsbad, CA, USA) was placed into a test tube and the magnetic beads were trapped by a magnet to separate the supernatant. After the supernatant was discarded, 1 mL P-16 mAb (0.5 mg/mL in 0.15 mol/L PBS, pH 7.8) was added to the magnetic beads and stirred at 25°C for 18 h. After washing the magnetic beads, 2 mL of 1% BSA in 0.15 mol/L PBS (pH 7.8) were added and stirred at 25°C for 18 h to block the beads. These beads were diluted to 1 mg/mL using the bead dilution reagent (0.05 mol/L Tris buffer (pH 7.5), 0.15 mol/L NaCl, 0.01% Tween 20, 0.1% NaN_3, 10% normal rabbit serum, and 0.1% mouse serum) when in use.

Ruthenium (Ru)-conjugated P-11 mAb was prepared by the following procedure: 68 μL Ru-complex compounds (10 mg Ru (II) Tris (bipyridyl)-NHS ester in 1 mL DMSO) was added

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to 1 mL P-11 mAb (1 mg/mL in 0.15 mol/L PBS, pH 7.8) (Sekisui Medical) for conjugation, and stirred at 25°C for 30 min. Then, 50 μL of 2 mol/L glycine was added to terminate the conjugation reaction, and Ru-conjugated P-11 mAb was isolated by collecting the Ru-bound protein fraction using Sephadex G-25 (previously equilibrated with 10 mmol/L PBS, pH 6.0). The Ru-conjugated P-11 mAb was then diluted to 1 μg/mL using Ru dilution reagent (0.015 mol/L HEPES buffering solution [pH 7.8], 0.15 mol/L NaCl, 0.013 mol/L $CaCl_2$, 0.1% Tween 20, 0.1% NaN_3, 5% normal rabbit serum, and 0.1% mouse serum) when in use.

Measurement of conventional DCP (with MU-3 antibody), NX-DCP (with P-11 and P-16 antibodies), and NX-DCP ratio. Conventional DCP, which is measured using MU-3 antibody and is currently used in clinical settings, was measured with ECLIA using the Picolumi III automated analyzer (EIDIA). NX-DCP was measured by ECLIA. Briefly, 25 μL magnetic beads coated with P-16 mAb (1 mg/mL) and 150 μL Ru-conjugated P-11 mAb (1 μg/mL) were added to samples at 30°C for 9 min to obtain the value of NX-DCP. The NX-DCP ratio was calculated by dividing the value of DCP measured using the conventional Picolumi method by the value of NX-DCP.

Reactivity of MU-3, P-11, and P-16 mAbs based on the time allowed for decarboxylation from prothrombin. We prepared DCP with varying numbers of Glu residues by applying different time intervals for decarboxylation from prothrombin (Enzyme Research Laboratories, Swansea, UK), according to the method of Bajah *et al.*[2] Specifically, 0.78 mL ammonium bicarbonate solution (0.1 mol/L, pH 8.0) was applied to 4.6 mg/mL prothrombin solution overnight for dialysis against an ammonium bicarbonate solution at 4°C. Then 0.1 mol/L EDTA*2Na was applied to the solution after dialysis until a final concentration of 10 mmol/L was achieved, and the solution was allowed to stand at room temperature for 30 min. This solution was dialyzed again against an ammonium bicarbonate solution for 2 h at 4°C then aliquoted into six heat-resistant vials with a screw cap and lyophilized. The vials were then filled with nitrogen gas and heated to 110°C for 0, 30 min, 1, 2, 6, or 23 h to create six different samples. We coated microplates with 100 μL of each sample at 0.1 μg/mL, and tested reactivity of the MU-3, P-11, and P-16 mAbs in the presence of 4 mmol/L calcium chloride. The experiments were repeated three times and the average value was calculated.

Patients. A total of 338 patients were diagnosed with primary, non-recurrent HCC between January 2006 and December 2009 at Ogaki Municipal Hospital (Ogaki, Japan). Of these, 14 patients had been taking warfarin when HCC was diagnosed. Six patients at Osaka Red Cross Hospital (Osaka, Japan) who were diagnosed as having primary HCC during the same period and had been taking warfarin at the time of diagnosis were also enrolled in the study. We analyzed the stored serum samples from these 20 patients, obtained at the time of HCC diagnosis. The diagnosis of HCC was made by histological examination or appropriate imaging characteristics using criteria of the guidelines by the American Association for the Study of Liver Diseases.[11] Tumor stage on imaging findings was assessed according to the TNM classification of the Liver Cancer Study Group of Japan.[12]

Control samples were obtained from 56 patients with chronic liver disease without HCC who were followed up at Ogaki Municipal Hospital. Samples were collected during routine HCC surveillance during the same period. These patients had been taking warfarin when serum samples were collected and provided informed consent for their stored serum samples to be used for research. The diagnosis of chronic liver disease was made with histological examination in 45 patients, including 10 with cirrhosis. The remaining 11 patients were diagnosed with cirrhosis based on imaging findings and biochemical tests. To ensure that controls did not have HCC, these patients were followed for 3 years after serum sampling by ultrasonography, CT, or MRI to ensure that none had developed HCC.

The protocol for the clinical part of this study was approved by the institutional review board of Ogaki Municipal Hospital and carried out in compliance with the Helsinki Declaration. Written informed consent was obtained from all study patients for the use of clinical and laboratory data and stored serum samples.

Statistical analyses. Differences in percentages between groups were analyzed using the χ^2-test. Differences in mean quantitative values were analyzed by the Mann–Whitney *U*-test. Changes in the NX-DCP ratio with increases in HCC stage were analyzed with the Jonckheere–Terpstra test. Data analyses were carried out using JMP statistical software, version 6.0 (Macintosh version; SAS Institute, Cary, NC, USA). All *P*-values were derived from two-tailed tests, with $P < 0.05$ considered to indicate statistical significance.

Results

Reactivity of MU-3, P-11, and P-16 antibodies with DCP based on time allowed for decarboxylation from prothrombin. Figure 1 shows the reactivity of the MU-3, P-11, and P-16 antibodies according to the time allowed for decarboxylation from prothrombin. MU-3 did not react with prothrombin (0 min) and its reactivity increased as the heating time (time allowed for decarboxylation) increased, with maximum reactivity to the sample after 6 h of heating. In contrast, P-11 and P-16 showed maximum reactivity to the 1-h sample and reactivity decreased as the heating time (time allowed for decarboxylation) increased.

Patient characteristics and levels of conventional DCP, NX-DCP, and NX-DCP ratio. Warfarin was used to treat atrial fibrillation in 48 patients, a history of mitral or aortic valve replacement in 13 patients, and a history of cerebral infarction in 15 patients. Table 1 summarizes the characteristics of the patients with and without HCC. There were no differences in patient

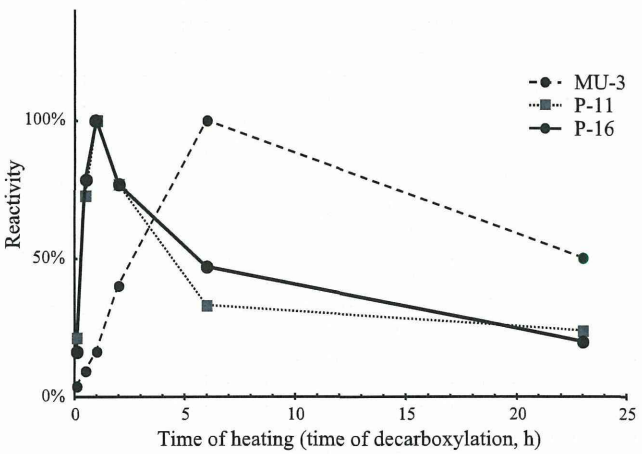

Fig. 1. Reactivity of MU-3, P-11, and P-16 antibodies according to the time allowed for decarboxylation from prothrombin. MU-3 did not react with prothrombin (0 min) and its reactivity increased as the heating time (time allowed for decarboxylation) increased, with maximum reactivity to the sample after 6 h of heating. Both P-11 and P-16 showed maximum reactivity to the 1-h sample and reactivity decreased as the heating time increased.

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Table 1. Background characteristics of study patients with and without hepatocellular carcinoma (HCC) (n = 76)

	Patients with HCC (n = 20)	Patients without HCC (n = 56)	P-value
Mean age ± SD, years (range)	72.4 ± 8.0 (46–83)	70.0 ± 9.8 (46–86)	0.3211
Sex, female/male	6 (30.0)/14 (70.0)	19 (33.9)/37 (66.1)	0.9651
Albumin, g/dL (mean ± SD)	3.82 ± 0.42	3.97 ± 0.51	0.2276
Total bilirubin, mg/dL (mean ± SD)	1.02 ± 0.65	0.82 ± 0.52	0.1288
Platelets ($\times 10^3/\mu L$)	158 ± 75	160 ± 49	0.4754
INR	1.75 ± 0.58	1.76 ± 0.58	0.7816
Mean tumor size ± SD, cm (range)	3.35 ± 1.84 (1.1–8.4)	–	–
Number of tumors, single/multiple	15 (75.0)/5 (25.0)	–	–
Tumor stage, I/II/III†	3 (15.0)/11 (55.0)/6 (30.0)	–	–

†According to the TNM classification of the Liver Cancer Study Group of Japan. Unless otherwise indicated, values in parentheses indicate percentages. INR, International normalized ratio.

age, sex, serum albumin, serum total bilirubin, platelet count, or prothrombin levels.

Figure 2 compares conventional DCP levels, NX-DCP levels, and NX-DCP ratios between patients with and without HCC. No differences were found in conventional DCP levels between patients with and without HCC (median, 2600.5 mAU/mL and range, 1060–96920 mAU/mL in patients with HCC versus median, 20550.5 mAU/mL and range, 1355–71783 mAU/mL in patients without HCC; $P = 0.7952$). In contrast, NX-DCP levels in patients with HCC (median, 34135.0 mAU/mL; range, 260–67581 mAU/mL) were significantly lower than in patients without HCC (median, 40708.0 mAU/mL; range, 5026–60443 mAU/mL; $P = 0.0291$). As a result, the NX-DCP ratio was significantly higher in patients with HCC (median, 1.92; range, 0.35–10.32) than in patients without HCC (median, 0.49; range, 0.12–1.33; $P < 0.0001$).

Sensitivity, specificity, and positive and negative predictive values of NX-DCP ratio for diagnosis of HCC. Figure 3(a) shows the receiver operating characteristic (ROC) curve of the NX-DCP ratio for the diagnosis of HCC. The area under the ROC curve was 0.8928. The highest Youden index was 0.68 when the cut-off was fixed as 0.65 and the highest accuracy was 89.5% when the cut-off was fixed as 1.50, based on the sensitivity and specificity analysis (Fig. 3b). When the cut-off was fixed as 0.65, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were 95.0%, 73.2%, 55.9%, 97.6%, and 78.9%, respectively. When the cut-off was fixed as 1.50, sensitivity, specificity, PPV, NPV, and accuracy were 60.0%, 100.0%, 100.0%, 87.5%, and 89.5%, respectively.

Serum alpha-fetoprotein (AFP) and *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3) levels in patients with HCC. Serum levels of AFP and AFP-L3 were measured[13] in patients with HCC in the same serum for the measurement of NX-DCP ratio (AFP-L3 was not measured in five patients). The median (range) values were 18.4 ng/mL (0.8–68 470 ng/mL) for AFP and 3.6% (0–45.2%) for AFP-L3. When the cut-off levels of AFP and AFP-L3 were fixed as 20 ng/mL and 5%, respectively, according to previous reports,[14–16] 10 of 20 patients (50.0%) showed elevation of AFP and seven of 15 patients (46.7%) showed elevation of AFP-L3. These two tumor markers were not increased in six of 15 patients (40.0%).

NX-DCP ratio and progression of HCC. Figure 4 shows the NX-DCP ratio in patients according to HCC stage. Despite the small number of patients, there was a gradual increase in the NX-DCP ratio as the stage increased ($P = 0.0315$).

Discussion

Hepatocellular carcinoma is the sixth most common cancer and the third most common cause of cancer-related death worldwide.[17,18] In Japan, HCC is currently the third most common cause of death from cancer in men and the fifth in women.[19] The incidence of HCC is also increasing in the US.[20,21] Improvements of tumor markers specific for HCC contribute to early detection of HCC. Three markers for HCC are currently used clinically, AFP, AFP-L3, and DCP. The utility of each of these tumor markers for detection and diagnosis of HCC, for evaluation of tumor progression, and for determination of patient prognosis has been reported.[4,22–24] Elevation

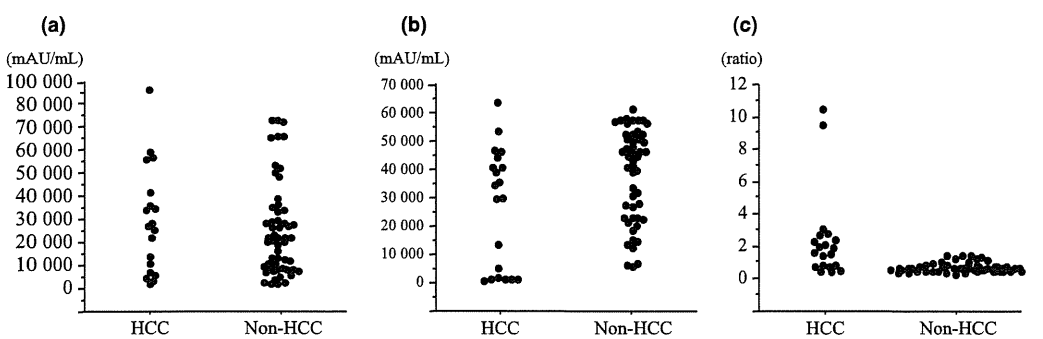

Fig. 2. Serum levels of conventional des-gamma-carboxy prothrombin (DCP), NX-DCP, and the NX-DCP ratio in patients with and without hepatocellular carcinoma (HCC) taking warfarin. (a) Serum levels of conventional DCP. No differences were found between two groups ($P = 0.7952$). (b) Serum levels of NX-DCP were significantly higher in patients without HCC compared to those with HCC ($P = 0.0291$). (c) The NX-DCP ratio was significantly higher in patients with HCC than in those without HCC, consequently ($P < 0.0001$).

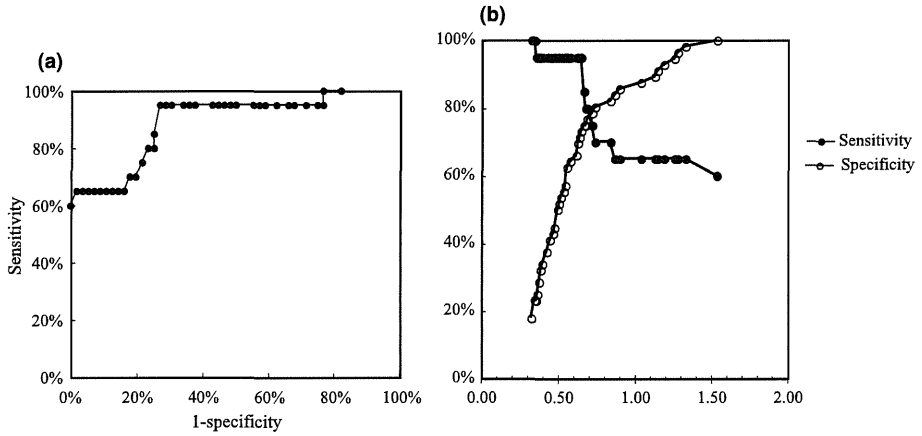

Fig. 3. Receiver operating characteristic (ROC) analysis and the determination of cut-off level of the NX-DCP ratio for the diagnosis of hepatocellular carcinoma. (a) The area under the ROC curve was 0.8928. (b) The highest Youden index was 0.68 when the cut-off was fixed as 0.65 and the highest accuracy was 89.5% when the cut-off was fixed as 1.50.

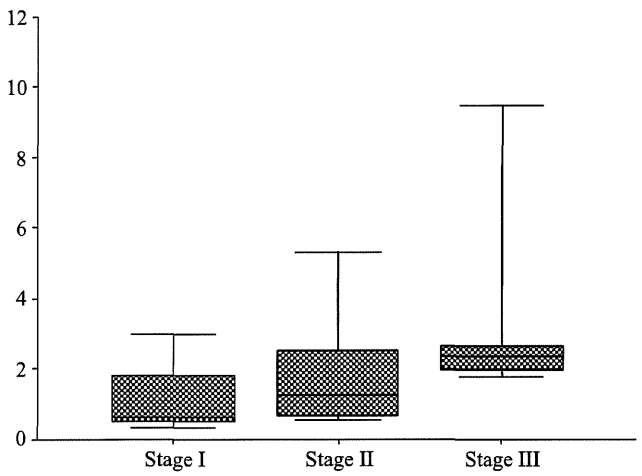

Fig. 4. The NX-DCP ratio according to hepatocellular carcinoma (HCC) stage in 20 patients with HCC taking warfarin (box plot). There was a gradual increase in the NX-DCP ratio as the HCC stage increased ($P = 0.0315$).

of DCP is often observed in HCC patients without elevation of AFP or AFP-L3, and is useful as a complement to these other two markers in the diagnosis of HCC. In addition, elevation of DCP is reportedly associated with a high rate of portal vein invasion and poor prognosis.[25] Elevation of DCP is also associated with better outcomes when hepatectomy, rather than radiofrequency ablation, is carried out in patients treated with curative intent.[26,27]

However, DCP loses its value as a tumor marker of HCC in patients taking warfarin.[7] Due to the marked decrease in vitamin K level caused by warfarin intake, DCP levels significantly increase in individuals taking warfarin, even in the absence of HCC. Therefore, DCP has no clinical utility as a tumor marker for HCC in this patient subpopulation.

The present study evaluated the reactivity of new antibodies against DCP, antibodies P-11 and P-16, based on the number of Glu residues. The number of Glu residues increases as the time allowed for decarboxylation from prothrombin increases.[2] Our results showed P-11 and P-16 have higher reactivity with DCP with fewer Glu residues than MU-3, the antibody that is conventionally used for the measurement of DCP. The NX-DCP level that is measured by P-11 and P-16 antibodies, therefore, represents predominantly DCP caused by reduced vitamin K availability. Consequently, the elevation of the NX-DCP ratio calculated in the equation: conventional DCP/NX-DCP, reflects more specifically the elevation of DCP by HCC.

There were no differences in the conventional measurements of DCP between patients with and without HCC who are taking warfarin. The NX-DCP ratio was significantly lower in patients without HCC than in patients with HCC. The NX-DCP ratio varied in patients with HCC, as was conventional DCP in patients not taking warfarin, because the production of DCP by HCC is variable. In contrast, in all patients without HCC, the NX-DCP ratio was low despite high conventional DCP levels in the same patients; no patients had an elevated NX-DCP ratio. The results indicate that the NX-DCP ratio could pinpoint the elevation of DCP caused by HCC, thereby restoring the value of DCP as a marker for HCC in patients taking warfarin.

When the cut-off level was fixed at 1.5 on the basis of maximal accuracy, the sensitivity, specificity, PPV, and NPV were comparable to those of conventional DCP in the general population with normal vitamin K levels, as previously reported.[28] The NX-DCP ratio, therefore, seems to be useful as a marker for HCC in patients taking warfarin.

The elevation of other tumor markers for HCC, AFP and AFP-L3, were observed in only half of the patients with HCC taking warfarin. In addition, both AFP and AFP-L3 were negative in 40% of patients with HCC. Des-gamma-carboxy prothrombin is a complimentary marker of AFP/AFP-L3 for HCC. The elevation of DCP without the elevation of AFP and AFP-L3 was observed in 16.1% of patients (cut-off, 20 ng/mL for AFP, 10% for AFP-L3, and 40 mAU/mL for DCP)[29] and 24.8% of patients (cut-off, 400 ng/mL for AFP, 15% for AFP-L3, and 100 mAU/mL for DCP).[30] The measurement of the NX-DCP ratio, therefore, will be important for the detection and diagnosis of HCC even when AFP or AFP-L3 is measured simultaneously.

There are several limitations to this study. The most important limitation was the small number of study patients, especially patients with HCC. The number of patients with HCC taking warfarin is low, so it was difficult to increase the number of study patients. Consequently, it was difficult to evaluate the value of the NX-DCP ratio in indicating progression of

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HCC, including tumor stage progression and portal vein invasion, and in predicting patient outcome. Further studies will be necessary to establish the value of the NX-DCP ratio as a tumor marker for HCC in patients taking warfarin. In addition, the value of the NX-DCP ratio was evaluated only in patients who were taking the vitamin K antagonist warfarin: its value was not evaluated in HCC patients in whom vitamin K is reduced or absent through other mechanisms such as heavy alcohol intake or nutritional deficiency. The value of the NX-DCP ratio as a marker for HCC should be confirmed for these subpopulations in the future.

In conclusion, the novel NX-DCP ratio identified elevation of DCP due to HCC in patients taking the vitamin K antagonist warfarin. Thus, by using this ratio, DCP can be used as a marker for HCC even in patients taking warfarin.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Long-term effect of lamivudine treatment on the incidence of hepatocellular carcinoma in patients with hepatitis B virus infection

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Abstract

Background Nucleotide analogues have recently been approved for the treatment of patients with hepatitis B virus (HBV) infection. However, it is still controversial whether the decrease of HBV-DNA amount induced by treatment with nucleotide analogues can reduce the risk of hepatocellular carcinoma (HCC) development in HBV patients.

Methods A total of 293 HBV patients without HCC who were treated with lamivudine (LAM) were enrolled in a multicenter trial. The incidence of HCC was examined after the start of LAM therapy, and the risk factors for liver carcinogenesis were analyzed. The mean follow-up period was 67.6 ± 27.4 months.

Results On multivariate analysis for HCC development in all patients, age ≥50 years, platelet count $<14.0 \times 10^4/mm^3$, cirrhosis, and median HBV-DNA levels of ≥4.0 log copies/ml during LAM treatment were significant risk factors. The cumulative carcinogenesis rate at 5 years was

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