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原著論文

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腹腔鏡下根治的膀胱摘除術の初期経験

要旨　【目的】膀胱癌に対する，腹腔鏡下根治的膀胱摘除術の当科における初期経験ならびに開腹術式との比較について報告する．

【対象と方法】2005年7月から2008年9月までの間に当科において腹腔鏡下根治的膀胱摘除術（LRC）を施行した24例と，ほぼ同時期に施行された開腹による根治的膀胱摘除術（ORC）16例を対象とした．LRCでの尿路変向術式は体外で行なった．また，尿路変向については，LRC，ORC双方ともに腸管を利用した術式（回腸導管または回腸新膀胱）を施行したものを対象とした．LRC群とORC群との比較を行い，LRC群の有用性について検討した．

【結果】術前因子（年齢，性別，Grade，臨床病期）はLRC群とORC群間に有意差を認めなかった．手術関連項目（手術時間，出血量，輸血率，術後イレウス発生率，術後経口摂取開始までの期間，術後合併症発生率，歩行開始までの期間，退院までの期間，術翌日の血中白血球数，CRP値）の比較においても今回の検討ではLRC群での明らかな優位性は認められなかった．病理学的所見，生存率（全生存率，非再発生存率）においても2群間の差を認めなかった．

【結論】尿路変向までを含めた場合，統計学的に明らかなLRCの有用性は確認できなかった．しかし，術中の拡大視野による正確な解剖の把握，良好な操作性，気腹による出血量の減少など，この術式の有用性は充分に期待できる．他の外科手技と同様，ラーニングカーブの影響をも鑑み，今後とも有用性についての検討をすすめる必要があると考えられた．

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Abstract　Objectives：Our initial experience in laparoscopic radical cystectomy or cystoprostatectomy (LRC) for bladder carcinoma in comparison with open radical surgery is reported.

Patients and Methods：Between July 2005 and September 2008, 24 patients underwent LRC followed by open urinary diversion. We compared peri- and postoperative findings of the 24 cases with those of 16 open radical cystectomy or cystoprostatectomy (ORC) conducted during the same time period. Urinary diversions in these patients were either ileal conduit (IC) or ileal neobladder (NB).

Results：There was no significant difference between LRC group and ORC group in terms of preoperative paramters (age, gender, tumor grade, clinical stage). Neobladder was more commonly chosen in LRC than in ORC. Statistically significant advantage was not evident in LRC group with regard to peri- and postoperative findings. Pathological findings and survival (overall, recurrence-free) were the same between two groups.

Conclusion：The advantage of LRC over ORC was not substantiated in our initial experience. Nevertheless, we consider this procedure as promising, which needs to be further pursued in terms of practical utility after learn-

ing curve being reached.

Key words：膀胱癌，腹腔鏡下根治的膀胱摘除術，周術期成績

緒　言

根治的膀胱摘除術は，膀胱癌（浸潤性癌，ハイリスク表在性癌）に対する標準治療である．開腹によるアプローチが標準術式であるが，泌尿器科領域における他臓器手術と同様，腹腔鏡下手技も取り入れられ，その短中期成績も検討されている．さらに最近ではロボット手術の経験も報告されている[1]ものの，改善の余地も多く，手技の標準化が期待されている．

本邦においても，徐々に報告[2-5]が増えてきており，今後，発展してゆくであろう分野の一つであると考える．

今回，我々の施設におけるLRCの初期経験と，同時期に施行されたORCとの比較検討につき報告する．

対象と方法

患者背景

2005年7月から2008年9月までの間に当科においてLRCを施行した症例で，腸管を利用した尿路変向術（回腸導管または回腸新膀胱造設）をおこなった24例と，同時期に施行されたORCの16例（同様に尿路変向については腸管を利用したもの）を対象とした．症例の詳細はTable 1に示す．

LRC群の年齢は平均64.3歳（48-84歳），男性21例，女性3例であった．臨床病期（T stage）ならびにgradeは，T1以下14例，T2：7例，T3：2例，T4（T4a）：1例，G1：1例，G2：4例，G3：17例，不明2例であった．尿路変向は回腸導管（Ileal conduit：IC）：7例，回腸新膀胱（Neobladder：NB）：17例であった．男性症例21例のなかで神経温存術を施行したものは2例，尿道摘除術を施行したものは4例（男性でIC6例中）であった．

ORC群の平均年齢は64.9歳（39-79歳），男性12例，女性4例であった．臨床病期（T stage）ならびにgradeは，T1以下5例，T2：10例，T3：0例，T4（T4a）：1例，G1：0例，G2:3例，G3:12例，不明1例であった．尿路変向はIC:13例，NB：3例であり，神経温存術施行例はなかった．男性でのIC 9例中，尿道摘除術は4例に施行された．

尿路変向は体外にて行い，NBは全例Studer 法で施行した．高齢，高度の合併症などのため，5例ではリンパ節郭清を施行せず原疾患の摘除にとどめた．術前補助化学療法（MEC療法）はORC群のT4症例（1例）のみで施行されていた．LRC群の1例では同時に腹腔鏡下での右腎摘除術が施行された．

LRC群とORC群について，臨床病理学的所見，また，手術関連項目として，手術時間，出血量，輸血率，術後イレウス発生率（イレウス管挿入やイレウス解除術を施行したもの），術後経口摂取開始（水分，食事）までの期間，術後合併症発生率（Minor：創哆開ならびに腎盂腎炎，発熱を伴うその他の炎症等で軽症なもの，Major：腸管吻合不全，重症感染症など，再手術，集中管理を要するような重症なもの），歩行開始までの期間，退院までの期間，術翌日の血中白血球数，CRP値などについて比較を行った．

統計処理はDr SPSS II®を用い，LRC群とORC群の2群間の比較を$\chi 2$検定（全生存率ならびに非再発生存率についてはlog-rank test）にて行なった．p値は0.05未満を有意

Table 1 Comparison of clinicopathological parameters between LRC and ORC

	LRC (%)	ORC (%)	p-value
Number pts	24	16	
Age (yo, mean ± SD)	64.3 ± 10.2	64.9 ± 11.2	NS
Gender			NS
male	21 (87.5)	12 (75)	
female	3 (12.5)	4 (25)	
Grade*			NS
1	1 (4.7)	0 (0)	
2	4 (19.0)	3 (20.0)	
3	17 (80.1)	12 (80.0)	
T stage			NS
≦2	21 (87.5)	15 (93.7)	
≧3	3 (12.5)	1 (6.2)	
Diversion			p=0.01
IC	7 (29.2)	13 (81.2)	
NB	17 (70.8)	3 (18.7)	
Nerve sparing**	2 (10.5)	0 (0)	NS
Urethrectomy***	4 (66.7)	4 (44.4)	NS

LRC：Laparoscopic radical cystectomy or cystoprostatectomy
ORC：Open radical cystectomy or cystoprostatectomy
IC：Ileal conduit　NB：Neobladder
NS；not significant, SD；standard deviation
*：Unassigned in 3cases（LRC：2, ORC：1）
：excluding female cases　*：Male pts, ileal conduit group

差ありとした.

LRC手術方法

手術は全身麻酔下に，頭低位，軽度開脚位（適時，切石位がとれるようにレビテーター®を使用）にて施行．まず，腹部にFig. 1のように5本のポートをおき，経腹膜的，順行性に膀胱摘除術を行う．以下，実際の手術方法につき概説する．

まず，腹腔内の観察を行い，操作に支障をきたすような癒着があれば，適時剥離しておく．次に，膀胱内に生理食塩水を約150ml注入し，腹膜越しに膀胱の輪郭を確認し，膀胱外側より約1cm外側で腹膜を縦切開する（Fig. 2）．下方に切開を進めると，この切開線に対し横切るように精管が確認されるので，この時点で切断する．さらに下方で，尿管が確認されるので，これを膀胱側に向かい剥離し（Fig. 3），尿管を切断し，断端は迅速病理診断に提出する．両側の尿管切断後，両側の腹膜の切開線をつなげるように，腹膜の横切開をおき，ここで，精嚢を確認し剥離する（Fig. 4）．lateral pedicleは超音波凝固切開装置（LCS）など用いて切断する．この後，直腸と前立腺の間の剥離，ならびに前立腺外側の処理を進める（この処理は腹腔鏡下前立腺全摘除術に準じて行う）．次に正中臍索を切断し，膀胱前腔を展開し，DVCの結紮を行う．神経温存は，当科では腹腔鏡下前立腺摘除術と同様にintra-fascial nerve sparing法にて施行している．尿道の切断は本検討では前立腺全摘除術と同様に行い，切断部をあらかじめ結紮する処理などは施行していない．また，尿道の処理は当初，開腹（下腹部正中切開）の後，膀胱を摘出する直前に行って

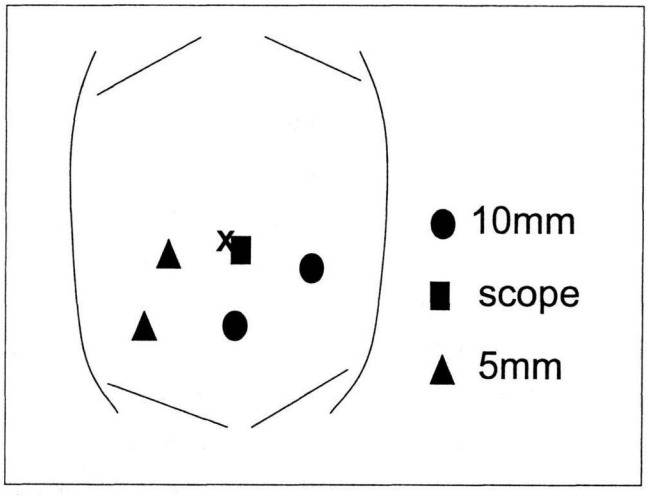

Fig. 1 ポートの位置

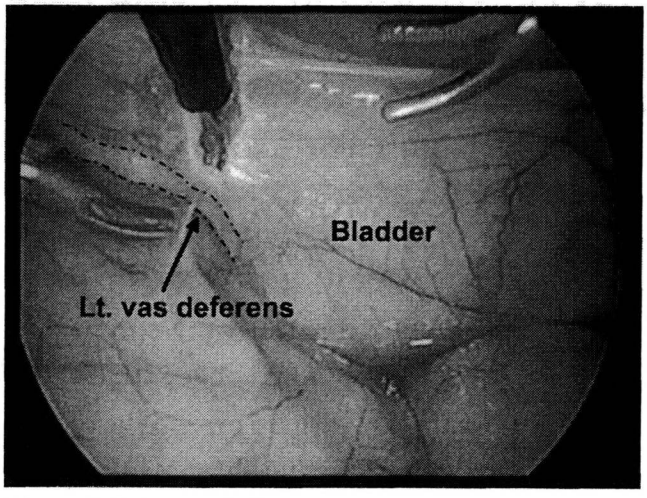

Fig. 2 膀胱左側での腹膜の切開

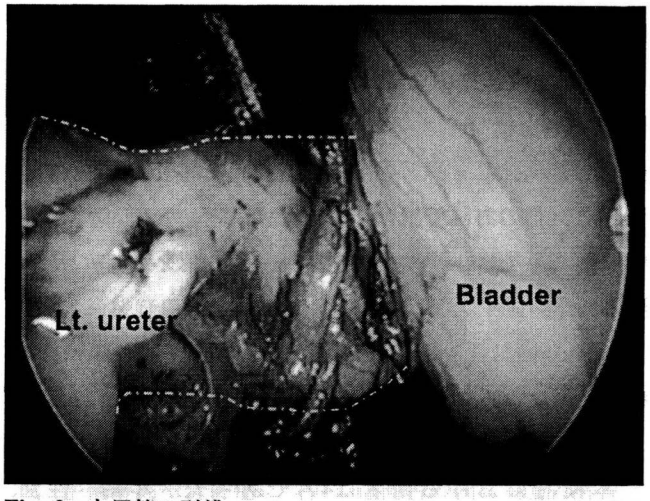

Fig. 3 左尿管の剥離

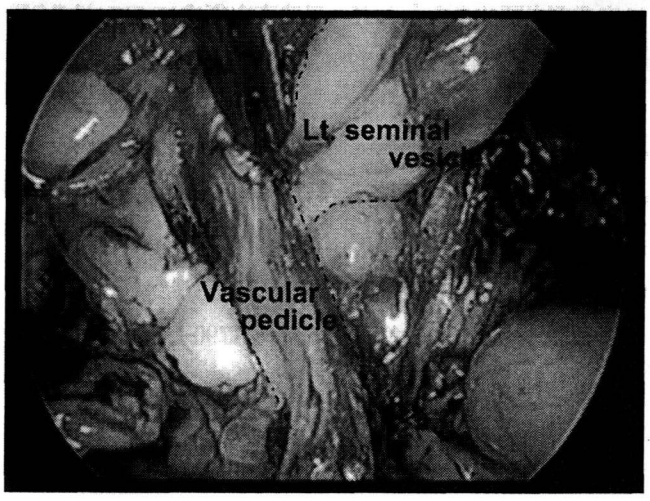

Fig. 4 左精嚢の剥離から血管茎の露出

Table 2 Peri- and postoperative findings of LRC and ORC

	LRC	ORC	p-value
Number pts	24	16	
Operative time (min)	620±122	477±68.5	NS
Blood loss* (ml)	1683±812	2350±1321	NS
Transfusion** (%)	62.5	75.0	NS
Transfusion*** (%)	37.5	68.7	p=0.053
Ileus (%)	16.7	18.7	NS
Oral intake			
Fluid (d)	6.0±4.1	4.0±0.8	NS
Diet (d)	8.6±6.4	6.8±2.6	p=0.02
Postop. complication			
Minor (%)	58.3	56.2	NS
Major (%)	12.5	6.2	NS
Ambulation (d)	2.8±1.2	2.6±0.9	NS
Hospital stay (d)	26.8±13.4	30.5±24.7	NS
WBC**** (/mm^3)	9996±2765	10219±3208	NS
CRP **** (mg/l)	8.61±1.90	9.70±2.02	NS

*: including urine
**: including autologous blood transfusion
***: excluding autologous blood transfusion
****: Data from day 1 following surgery
Figures are mean±standard deviation.

Table 3 Pathological findings and survival in LRC and ORC

	LRC	ORC	p-value
Number of pts	24	16	
Grade*			NS
G2 (%)	5 (25.0)	3 (23.1)	
G3 (%)	15 (75.0)	10 (77.9)	
pT stage			NS
≦2 (%)	15 (62.5)	8 (50.0)	
≧3 (%)	9 (37.5)	8 (50.0)	
Number of lymph nodes (mean±SD) **	14.6±6.0	17.8±10.3	NS
Lymph node mets. (%) **	5 (22.7)	4 (30.8)	NS
Pos. surgical margin (%)	0 (0)	1 (6.25)	NS
Follow- up (d, mean±SD)	504±297	311±293	NS
Survival			
Overall (%)	21 (87.5)	14 (87.5)	NS
Recurrence-free (%)	21 (87.5)	12 (75.0)	NS

pT0 were 5 cases (LRC : 4, ORC1) in this study.
* : excluding 2 cases (ORC : 2) of non-urothelial carcinoma
** : excluding 5 cases (LRC : 2, ORC : 3) without lymph node dissection.
SD, standard deviation

いた.（下腹部正中切開はカメラのポートと正中の10mmのポートを結び，さらに恥骨上までの皮膚切開とした.）組織の摘出，リンパ節郭清，尿路変向は開腹後に通常の開腹術と同様の方法で施行した.

尚，本手術は，学内の倫理委員会の承認を得て実施している.

結　果

本検討における術者は4名であった．年齢，性別，grade，臨床病期（T stage）についてLRC群とORC群間に有意差を認めなかったが，尿路変向術式につてはLRC群において回腸新膀胱が，ORC群において回腸導管がより多く選択（p=0.01）されていた（Table 1）.

LRC群とORC群別の手術関連項目，病理学的所見，生存率の結果について示す（Table 2, 3）．手術時間はLRC群が平均620分（337－800分），ORC群が477分（360－585分）であった（p=0.3）．出血量はLRC群が平均1683ml (380-3295ml), ORC群が平均2350ml (790-5700ml) であった（p=0.4）．自己血輸血を含む輸血を要したものはLRC群が15例（62.5%），ORC群が12例（75.0%）であり（p=0.4），同種血輸血をおこなったものはLRC群が9例（37.5%），ORC群が11例（68.7%）であった（p=0.053）．術後イレウスをLRC群では4例（16.7%）に，ORC群では3例（18.7%）に認めた（p=0.8）．経口摂取開始（飲水，食事）までの期間は，それぞれLRC群で平均6.0日（3－23日），8.6日（6－37日），ORC群が平均4.0日（3－5日），6.8日（4－13日）であった（各p=0.3, 0.02）．Minor，Majorな術後の合併症発生率は，それぞれLRC群が14例（58.3%），3例（12.5%），ORC群が9例（56.2%），1例（6.2%）であった（各p=0.4, 0.8）．腸管吻合不全に伴うイレウスはイレウス発生と術後合併症（Major）ともに「あり」と分類した．合併症の種類と症例の背景をTable 4 (a, b) に示す（症例は手術施行順に記した）．創哆開については，軽度のものもすべて含めた．今回の検討ではMajorなものは4例（ORC群1例，LRC群3例）で，すべて腸管吻合不全であった．4例中3例（ORC群1例，LRC群2例）は手術にて修復した．LRC群の1例は保存的に治療しえたが，食事の開始まで37日を要した．術後歩行開始までの期間はLRC群が平均2.8日（1－6日），ORC群が平均2.6日（1－5日）であった（p=0.1）．退院までの期間はLRC群が平均26.8日（14－83日），ORC群が平均30.5日（16－120日）であった（p=0.5）．手術翌日の血中白血球数，CRP値はそれぞれLRC群が平均9996/mm^3（5600－16400/mm^3），平均8.61mg/l（5.81－13.27mg/l），ORC群が平均10219/mm^3（4200－18600/mm^3），平均9.70mg/l（5.27－12.61mg/l）であった（各

Table 4a Complication in ORC group

Age (yo)	Gender	Stage	Diversion	Op. time (min.)	Blood loss (ml)	Complication
70	M	T1	IC	505	790	WI
76	M	T2	IC	425	1232	WD
66	F	T2	IC	490	2120	WD
75	M	T1	IC	520	2810	WD
						ABL*
66	M	T4	IC	360	1900	PN
39	M	T2	NB	540	4420	PN
77	F	T2	IC	510	2250	PN
59	F	T2	IC	480	3880	WD
67	M	T2	IC	509	1790	WD

*: Major complication
WI: Wound infection WD: Wound dehiscence
ABL: Anastomotic bowel leak PN: Pyelonephritis

Table 4b Complication in LRC group

Age (yo)	Gender	Stage	Diversion	Op. time (min.)	Blood loss (ml)	Complication
56	M	T1	NB	800	2695	PN
58	M	T2	NB	740	1430	PN
62	M	T2	NB	760	2000	WD
						PN
75	M	T3	NB	615	2810	WD
60	M	T1	NB	655	2680	WD
76	M	T1	IC	610	2317	WD
48	M	T1	NB	480	1560	WD
63	M	T2	NB	630	1712	PN
58	M	T2	NB	540	1790	EP
79	M	T1	IC	632	1550	FUO
54	M	T2	NB	580	610	WD
						ABL*
84	M	T1	IC	337	380	PN
64	M	T3	IC	395	1170	ABL*
61	M	T1	IC	680	1420	WD
						ABL*,**
58	F	T1	NB	622	1560	WD

*: Major complication
**: Treated conservatively
PN: Pyelonephritis WD: Wound dehiscence EP: Epididymitis
FUO: Fever of unknown origin ABL: Anastomotic bowel leak

p=0.4, 0.4).

上述のように，食事の開始についてはORC群で有意（p=0.02）に短かった．同種血輸血を行った率では有意差は得られなかったがLRC群で少ない（LRC群では自己血のみでコントロール可能な率が高い）傾向があった（p=0.053）．そのほかの項目については2群の間に有意差は認めらなかった．

両群の病理学的所見（Grade，pT stage，リンパ節転移陽性率，切除断端陽性率）に有意差を認めなかった（各p=0.9, 0.4, 0.3, 0.2）．pT0 は5例（LRC：4例，ORC：1例）に認めた．悪性度（Grade）の比較においては腺癌（ORC：1例），扁平上皮癌（ORC：1例）は除外した．

シスプラチンを用いた術後補助化学療法（MEC療法）はLRC群7例，ORC群2例に対して行われたが有意差は認めなかった（p=0.2）．

術後観察期間はLRC群が平均504日（19－1089日），ORC群が平均311日（50－1128日）で有意差を認めず（p=0.4），観察期間は短いが，生存率（全生存率，非再発生存率）においてもこの2群間に差を認めなかった（p=0.4, 0.08）．

なお，LRC群において2例で術中に直腸損傷を認めたが，術中に修復し得た．術後，直腸損傷による合併症は認めなかった．

考　察

ORCとLRC＋体外での尿路変向術の周術期成績に関する比較について，Haberら[1]はLRC＋体外での尿路変向術では出血量やイレウスの発生がより少なく，入院期間が短いことを報告している．また，Basillotеら[6]はORCに比較してLRC＋体外での尿路変向術は術後の鎮痛剤の使用量が少なく，術後経口摂取開始までの期間が短く，入院期間や軽作業への復帰までの期間が短いこと，さらにPropigliaら[7]はLRCのグループでは鎮痛剤使用がより少なく，術後経口摂取可能となるまでがより短いことを報告している．そして，手術時間や合併症については両者の間に有意な差を認めなかった[1,6]としている．

また，尿路変向についてHaber[8]らは，体外で行うOpen-assistedの方が，腹腔鏡下で行う方法（Pure laparoscopic）よりも手術時間が短く，出血量と輸血率が少なく，術後の経口摂取可能となるまでの期間，入院期間が短いとしている．小さな合併症についてはPure laparoscopicの方が多く，再手術を要するような合併症の発生率はPure laparoscopicでは29％，Open-assistedでは11％（p=0.08）と報告している．現時点では，LRCはORCよりもメリットがあるが，尿路変向については体外で行うほうがよいという結論である．

今回のわれわれの検討では上に挙げた報告例のような明らかな有用性は認められなかった．今回の検討ではLRC

とORCの症例において選択された尿路変向法に差があったことが要因となった可能性もある．出血量に関しては，LRCでは膀胱全摘（気腹終了時）までの平均値は217.5ml（30-650ml）と比較的少量にコントロールされていた．しかし，開腹術に移行してからのoozingなどにより全体の出血量が増加してしまったと考えられる．食事開始までの期間について，今回の検討ではORC群の方が短期間であったが，LRC群においては腸管吻合不全により37日を要した症例もあり，少ない症例数での比較において影響が大きかった可能性もある．低侵襲性の評価については，今回の検討では術翌日の血中白血球数とCRP値，歩行開始までの期間を指標としたが，いずれも有意差を認めなかった．今後は海外での報告例のように術後鎮痛剤の投与量や投与期間などについても評価を行う必要があると考える．

尿路における他臓器の腹腔鏡下手術と同様に，この術式においても，腹腔鏡下での拡大視野のもとでの操作，気腹による出血量の軽減，低侵襲化などのメリットが期待される．さらに腸管を利用した尿路変向において，腸管の浮腫の軽減も期待でき，術後の消化管機能回復期間の短縮が期待される．また，今回の検討では，術者が4名で，症例数も24例と少なく，データには示していないが明らかなラーニング効果は認められなかった．しかし，他の外科手術と同様に，今後ラーニングカーブが立ちあがり，安定することも十分に期待できる．このような点からも，さらに症例を増やし，この術式と開腹術との比較検討を進めていく予定である．

結　語

当科で施行したLRCの初期経験ならびに，同時期に施行された開腹による根治的膀胱摘除術との比較について報告した．今回の検討では，諸種所見の数値上，海外での報告のような有用性は認められなかったものの，十分に今後が期待できる手術手技であると考えられた．

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Research Article

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Association between mutations in the core region of hepatitis C virus genotype 1 and hepatocellular carcinoma development

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Background & Aims: To determine whether amino acid mutations in the core region of hepatitis C virus (HCV) genotype 1 are associated with response to interferon (IFN) therapy and development of hepatocellular carcinoma (HCC).

Methods: We followed up 361 patients (median duration, 121 months), and IFN monotherapy was administered to 275 (76%) [sustained virological response (SVR) rate, 26.5%]. Using pretreatment sera, mutations at core residues 70 and 91 were analyzed [double wild (DW)-type amino acid pattern: arginine, residue 70; leucine, residue 91].

Results: A low aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio and low HCV load were independently associated with SVR, but core mutations were not. During follow-up, 12 of 81 (14.8%) patients with the DW-type pattern and 52 of 216 (24.1%) patients with non-DW-type pattern developed HCC ($p = 0.06$, Breslow–Gehan–Wilcoxon test). Multivariate analysis with the Cox proportional-hazards model revealed the following independent risk factors for HCC: male gender [$p < 0.0001$; risk ratio (RR), 3.97], older age ($p < 0.05$; RR, 2.08), advanced fibrosis ($p < 0.0001$; RR, 5.75), absence of SVR ($p < 0.01$; RR, 10.0), high AST level ($p < 0.01$; RR, 2.08), high AST/ALT ratio ($p < 0.01$; RR, 2.21), and non-DW-type pattern ($p < 0.05$; RR, 1.96). In patients with F0–F2 fibrosis at entry, non-DW-type was likely to lead to cirrhosis ($p = 0.051$).

Conclusions: In HCV genotype 1 patients, HCC risk could be predicted by studying core mutations, response to IFN, and host factors like age, gender, and liver fibrosis.

Keywords: Hepatitis C virus; Core region; Hepatocellular carcinoma; Interferon; Sustained virological response.

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Abbreviations: HCV, hepatitis C virus; IFN, interferon; HCC, hepatocellular carcinoma; SVR, sustained virological response; DW-type, double wild-type; RR, risk ratio; AST, aspartate aminotransferase; ALT, alanine aminotransferase; US, ultrasonography; CT, computed tomography; MRI, magnetic resonance imaging; PCR, polymerase chain reaction; OR, odds ratio.

Introduction

Hepatitis C virus (HCV) infection is a global health problem and the number of chronic carriers worldwide is estimated at 170 million [1]. HCV causes chronic hepatitis, which may progress to liver cirrhosis and hepatocellular carcinoma (HCC); the speed of disease progression, though, varies among patients [2,3]. Age, gender, steatosis, liver fibrosis, and response to interferon (IFN) therapy are reported to be associated with disease progression and HCC development [4–7]. HCV has six major genotypes, of which genotype 1 is most common in Japan and reported to be associated with increased severity and progression of chronic liver disease [8,9]. HCV contributes to HCC by directly modulating the pathways promoting the malignant transformation of hepatocytes [10–13]. Studies on transgenic mice revealed that the HCV core protein has oncogenic potential [14], but other studies yielded conflicting results [15,16]. Recently, mutations at amino acids 70 and 91 in the core region were shown to predict virological response to therapy with IFN plus ribavirin and also HCC development [17–19]. However, few studies support these results, and hence, the clinical impact of core mutations on HCC development is still unclear. In order to determine the viral factors associated with HCC development, we performed a retrospective cohort study on 361 patients with chronic liver disease caused by HCV genotype 1 infection and analyzed the amino acids present at core residues 70 and 91. Additionally, we evaluated whether these mutations were associated with IFN treatment, cirrhosis development, or host factors like age and gender.

Patients and methods

Study population

We enrolled 361 consecutive HCV genotype 1-infected patients who had undergone liver biopsy between August 1986 and June 1998 at Chiba University Hospital. At the enrollment time, the absence of HCC was proven by abdominal ultrasonography (US), computed tomography (CT), or magnetic resonance imaging (MRI). All the patients tested positive for anti-HCV antibody, determined by second-generation enzyme-linked immunosorbent assay. Patients with chronic hepatitis B, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, Wilson disease, or alcoholic liver disease were excluded, as were patients with a history of alcoholism, drug abuse, or IFN therapy. Written informed consent was obtained from all patients before performing liver biopsy.

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Table 1. Baseline characteristics of 361 hepatitis C (HCV) genotype 1-infected patients according to hepatocellular carcinoma (HCC) development.

Patients	$n = 361$	HCC development		
		(+), $n = 82$	(−), $n = 279$	p value
Gender (male/female)	219/142	56/26	163/116	0.1
Age (years)	50.5 ± 12.2	56.8 ± 7.1	48.6 ± 12.7	<0.0001
BMI (kg/m^2)	23.1 ± 2.9	23.1 ± 2.8	23.1 ± 3.3	0.82
Staging of fibrosis (F0–1/F2/F3/F4)	197/59/52/53	13/18/23/28	184/41/29/25	<0.0001
IFN treatment and response				
SVR/non-SVR/non-IFN	73/202/86	4/55/23	69/147/63	0.0004
Laboratory data				
AST (IU/L)	87 ± 62	109 ± 59	80 ± 61	0.0001
ALT (IU/L)	125 ± 93	139 ± 80	121 ± 96	0.13
AST/ALT	0.75 ± 0.26	0.84 ± 0.28	0.73 ± 0.25	0.0003
Platelets (10^4/mm^3)	17.7 ± 6.7	13.0 ± 3.3	18.2 ± 6.9	<0.0001
Albumin (g/dL)	4.2 ± 0.36	4.1 ± 0.39	4.3 ± 0.35	<0.0001
Total bilirubin (mg/dL)	0.8 ± 0.6	0.9 ± 0.3	0.8 ± 0.6	0.39
Core protein (pg/mL)	201 ± 245	283 ± 273	177 ± 231	0.001
Amino acid pattern				
70 Wild/non-wild/ND	168/129/64	32/32/18	136/97/46	0.23*
91 Wild/non-wild/ND	139/158/64	28/36/18	111/122/46	0.58*
DW/non-DW/ND	81/216/64	12/52/18	69/164/46	0.08*

BMI, body mass index; DW, double wild (arginine at residue 70 and leucine at residue 91 in the core region); ND, not detected; ND cases were excluded.

The clinical backgrounds of the patients are shown in Table 1. The study population was predominantly male (59% men), and the mean age of the patients was 50.5 ± 12.2 years, with 15% patients having liver cirrhosis.

Laboratory examination

Serum samples were obtained and stored at −30 °C until analysis. We assumed that genotype 1 corresponds to group 1 when determining the HCV RNA genotypes by serologic grouping of serum antibodies [20]. The serum HCV load of the patients was determined at the time of liver biopsy, using the HCV core protein detection kit (Eiken Chemical, Tokyo, Japan; detection limit, 8 pg/mL) [21].

Histopathological examination

Percutaneous liver biopsy was performed, and specimens were histopathologically assessed as described previously [22]. According to the criteria of Desmet et al. [23], the staging of fibrosis was defined as F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis), and F4 (cirrhosis).

Core nucleotide sequences

HCV RNA was extracted from the serum samples obtained at the time of liver biopsy, and it was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Nucleic acids were amplified by PCR with the

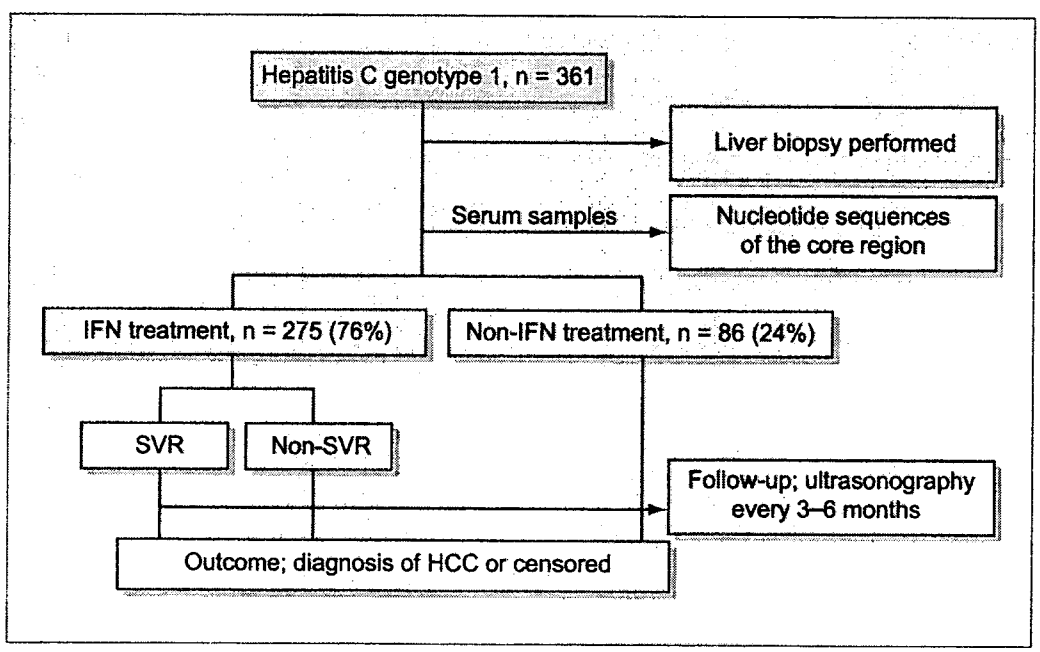

Fig. 1. Clinical courses after enrollment and the evaluation methods. IFN, interferon; SVR, sustained virological response; HCC, hepatocellular carcinoma. [This figure appears in colour on the web.]

HotStart Taq Master Mix kit (Qiagen, Hilden, Germany) and primers that have been previously described [24]. Polymerase chain reaction (PCR) was initiated with a denaturation step at 95 °C for 15 min, followed by 45 cycles at 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 3 min, and subsequent extension for 7 min. PCR products were resolved by agarose gel electrophoresis, purified using the QIA quick PCR purification kit (Qiagen), and directly sequenced using a Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Tokyo, Japan). The sequences were determined using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

As described previously, the double wild-type (DW-type) amino acid pattern was defined as the presence of arginine at residue 70 (wild-type) and leucine at residue 91 (wild-type) [19].

IFN treatment

Depending on whether IFN was administered, the patients were divided into the IFN (76%) and non-IFN groups (24%) (Fig. 1). Patients who received IFN monotherapy during follow-up were divided into two subgroups: the sustained virological response (SVR) group, including patients who tested negative for HCV RNA at 24 weeks after completion of therapy, and non-SVR group (Fig. 1). Of the 275 patients in the IFN group, 73 (26.5%) achieved SVR.

Follow-up and diagnosis of cirrhosis and HCC

Clinical assessments were performed at least once every month during IFN treatment and every 3–6 months after the treatment. During follow-up, abdominal US was performed every 3–6 months to determine whether HCC had developed (Fig. 1). If necessary, additional procedures like CT, MRI, abdominal angiography, and US-guided tumor biopsy were performed to confirm HCC development. We also evaluated whether cirrhosis had developed in non-cirrhotic patients (F0–F2 stage). Cirrhosis was diagnosed according to the criteria of cirrhosis as described previously [25,26]. The follow-up period was the duration from the initial liver biopsy to HCC diagnosis or the last follow-up visit. For non-cirrhotic patients, this was the duration from the start point to cirrhosis diagnosis.

Statistical analysis

The χ^2 test was used to compare categorical variables, and Student's t test to compare continuous variables related to background characteristics among groups. Continuous variables were expressed as mean ± standard deviation. The cumulative incidence of HCC and cirrhosis was calculated using the Kaplan–Meier method and evaluated using the Breslow–Gehan–Wilcoxon test. Multivariate analysis was performed using the Cox proportional-hazards model or multiple logistic regression analysis. The Cochran–Armitage trend test was used for analyzing the association between the prevalence of mutation and subject age. Statistical significance was defined as $p < 0.05$.

Results

Cumulative HCC incidence

During follow-up (median duration, 121 months; range, 8–257 months), 82 (22.7%) patients developed HCC [HCC group; 13 of 197 (6.6%) from F0–F1, 18 of 59 (30.5%) from F2, 23 of 52 (44.2%) from F3, and 28 of 53 (52.8%) from F4 stage at entry] and 279 (77.3%) did not (non-HCC group). The cumulative HCC incidence at 5, 10, and 15 years of follow-up was 9.5%, 22.9%, and 30.9%, respectively.

Core nucleotide sequences

The core nucleotide sequence was determined for 297 of 361 (82.3%) patients. In the entire patient group, the proportions of DW-type and non-DW-type patterns were 22% and 60%, respectively (Table 1).

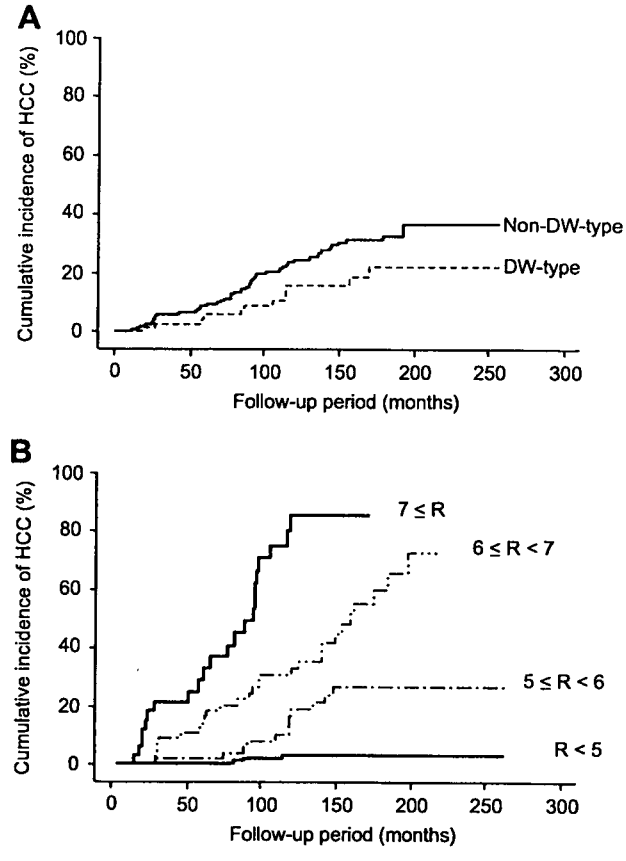

Fig. 2. Cumulative incidence of hepatocellular carcinoma (HCC) in hepatitis C genotype 1-infected patients. (A) Comparison between patients with double wild-type (DW-type: arginine, residue 70; leucine, residue 91) ($n = 81$) and non-DW-type ($n = 216$) amino acids in the core region ($p = 0.06$). (B) Comparison based on risk score (R) calculated using independent variables for HCC risk ($p < 0.0001$).

The core nucleotide sequence could not be determined for 64 patients because their samples showed significantly lower levels of the HCV core protein than those obtained from the 297 patients in whom the core sequence could be detected (119 vs. 217 pg/mL; $p = 0.0083$). There was no significant difference between the other variables shown in Table 1.

Cumulative HCC incidence according to core amino acid mutations

During follow-up, 12 of 81 (14.8%) patients with the DW-type pattern and 52 of 216 (24.1%) patients with the non-DW-type pattern developed HCC. Cumulative HCC incidence was 6.8% and 11% at 5 years, 19.1% and 27.7% at 10 years, and 26.6% and 38% at 15 years in the DW-type and non-DW-type groups, respectively. Cumulative HCC incidence in the DW-type group tended to be lower than that in the non-DW-type group ($p = 0.06$; Fig. 2A).

Predictive factors associated with HCC development

Potential predictive factors associated with HCC development are shown in Table 1. Univariate analysis revealed 10 parameters correlating with HCC development (Table 1). Multivariate analy-

Table 2. Factors associated with hepatocellular carcinoma development in hepatitis C genotype 1-infected patients, identified by multivariate analysis using the Cox proportional-hazards model.

Factor*	Category	Risk ratio (95% CI)	*p* value
Gender	Male	3.97 (2.05–7.63)	<0.0001
	Female	1.0	
Age (years)	⩾50	2.08 (1.01–4.33)	0.049
	<50	1.0	
Staging of fibrosis	⩾2	5.75 (2.68–12.35)	<0.0001
	<2	1.0	
IFN treatment and response	Absence of SVR	10.0 (2.29–43.48)	0.002
	SVR	1.0	
AST (IU/L)	>90	2.08 (1.20–3.62)	0.009
	⩽90	1.0	
AST/ALT	⩾0.8	2.21 (1.24–3.97)	0.007
	<0.8	1.0	
Amino acid pattern	Non-DW	1.96 (1.02–3.76)	0.04
	DW	1.0	

CI, confidence intervals; DW, double wild (arginine at residue 70 and leucine at residue 91 in the core region).
*Significant factors are shown.

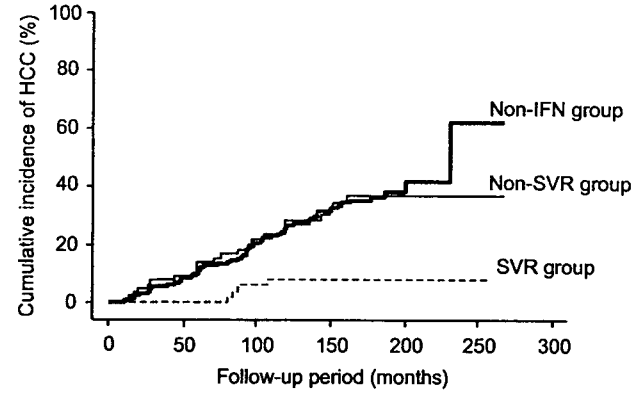

Fig. 3. Cumulative incidence of hepatocellular carcinoma (HCC). Comparison between the sustained virological response (SVR) ($n = 73$), non-SVR ($n = 202$), and non-interferon (IFN) ($n = 86$) groups ($p = 0.002$).

sis with the Cox proportional-hazards model showed that the following seven independent parameters were significantly associated with HCC development: male gender ($p < 0.0001$), age ⩾ 50 years ($p = 0.049$), fibrosis ⩾ F2 ($p < 0.0001$), absence of SVR ($p = 0.002$), aspartate aminotransferase (AST) level > 90 IU/L ($p = 0.009$), AST/alanine aminotransferase (ALT) ratio ⩾ 0.8 ($p < 0.007$), and non-DW-type pattern in the core region ($p = 0.04$) (Table 2).

Prediction of HCC development based on risk score

Using the predictive variables from the previous step (Table 2), the risk score (R) for HCC development was calculated from the beta coefficients derived from the Cox proportional-hazards model as follows: $R = 0.671 \times$ (non-DW-type) $+ 2.307 \times$ (absence of SVR) $+ 0.733 \times$ (AST > 90 IU/L) $+ 0.733 \times$ (age ⩾ 50 years) $+ 1.752 \times$ (staging of fibrosis ⩾ 2) $+ 1.378 \times$ (male) $+ 0.795 \times$ (AST/ALT ⩾ 0.8) (each variable: yes = 1, no = 0). Fig. 2B shows the cumulative HCC incidence of four subgroups categorized by risk score, and the RR of each group is shown in Table 3. The cumulative HCC incidence increased with the risk score: from highest to lowest it was 84.7%, 35.1%, 18.5%, and 3.0% at 10 years.

Cumulative HCC incidence according to IFN treatment and response

During follow-up, 4 (5.5%) patients in the SVR, 55 (27.2%) in the non-SVR, and 23 (26.7%) in the non-IFN groups developed HCC; cumulative HCC incidence was 0%, 11.3%, and 13.2%, respectively, at 5 years; 7.8%, 25.6%, and 27.3%, respectively, at 10 years; and 7.8%, 36.5%, and 35.5%, respectively, at 15 years. Moreover, cumulative HCC incidence was significantly lower in the SVR group than other groups ($p < 0.001$; Fig. 3).

Table 3. Relative risk of HCC development based on risk score, using the Cox proportional-hazards model.

Score (R)	Risk ratio (95% CI)	*p* value
$R < 5$	1	
$5 \leqslant R < 6$	9.22 (2.60–32.7)	0.0006
$6 \leqslant R < 7$	26.9 (8.15–89.0)	<0.0001
$7 \leqslant R$	88.3 (25.8–302)	<0.0001

CI, confidence intervals.

Analysis of SVR-associated factors

Compared to those in the non-IFN group, patients in the IFN group were younger (49 years vs. 54 years, $p = 0.003$), had higher aminotransferase levels (AST, 93 vs. 68 IU/L, $p = 0.001$; ALT, 137 vs. 87 IU/L, $p < 0.0001$) and lower core protein levels (183 vs. 263 pg/mL, $p = 0.01$). Table 4 shows baseline characteristics of patients according to interferon response. Univariate analysis revealed six SVR-associated parameters, whereas multiple logistic regression analysis revealed two independent significant predictors of SVR: AST/ALT ratio of <0.8 [$p = 0.005$; odds ratio (OR), 3.09; 95% confidence interval (CI), 1.40–6.82] and core protein level of <200 pg/mL [$p < 0.0001$; OR, 70.94; 95% CI, 9.56–526.2]. However, both univariate ($p = 0.64$) and multivariate analyses (data not shown) showed that the DW-type pattern in the core region was not associated with SVR.

Table 4. Baseline characteristics of patients according to interferon response.

Nature of the Regime	SVR $n = 73$	Non-SVR $n = 202$	*p* value
Gender (Male/Female)	47/26	126/76	0.76
Age (years)	46.6 ± 13.3	50.5 ± 11.5	0.02
BMI (kg/m^2)	22.7 ± 2.8	23.2 ± 3.0	0.24
Staging of fibrosis: (F0-1/F2/F3/F4)	45/12/9/7	104/34/34/30	0.42
Laboratory data			
AST (IU/L)	79 ± 56	97 ± 69	0.048
ALT (IU/L)	132 ± 92	139±100	0.60
AST/ALT	0.65 ± 0.22	0.75 ± 0.27	0.003
Platelets (10^4/mm^3)	18.6 ± 6.7	16.7 ± 6.1	0.03
Albumin (g/dL)	4.3 ± 0.3	4.2 ± 0.4	0.06
Total bilirubin (mg/dL)	0.7 ± 0.4	0.8 ± 0.4	0.02
Core protein (pg/mL)	31 ± 50	234 ± 226	<0.0001
Amino acid pattern			
70 Wild/Non-wild/ND	35/21/17	89/74/39	0.30
91 Wild/Non-wild/ND	24/32/17	76/87/39	0.62
DW/Non-DW/ND	14/42/17	46/117/39	0.64

BMI, body mass index; DW, double wild (arginine at residue 70 and leucine at residue 91 in the core region); ND, not detected.

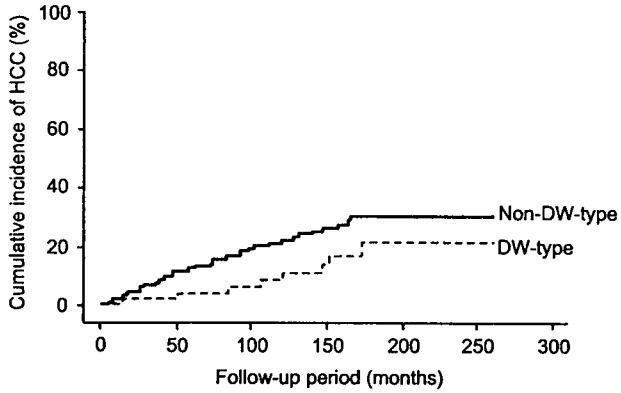

Fig. 4. Cumulative incidence of cirrhosis in non-cirrhotic patients (F0–F2). Comparison between patients with double wild-type (DW-type: arginine, residue 70; leucine, residue 91) ($n = 81$) and non-DW-type ($n = 216$) amino acids in the core region ($p = 0.051$).

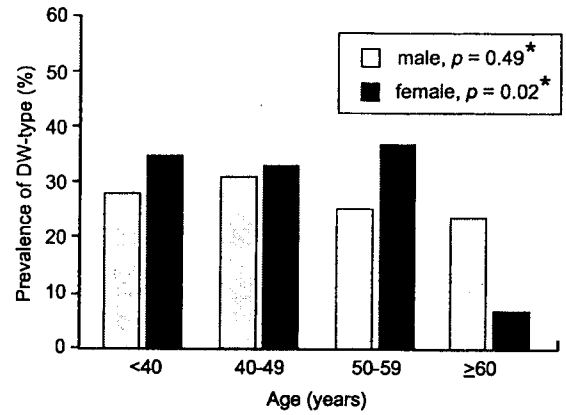

Fig. 5. Prevalence of double wild-type (DW-type: arginine, residue 70; leucine, residue 91) amino acids in the hepatitis C core region according to age and gender. *By the Cochran-Armitage trend test.

Cumulative cirrhosis incidence for non-cirrhotic patients (F0–F2)

Of the 256 non-cirrhotic patients (197 from F0–F1, 59 from F2), 50 (19.5%) developed cirrhosis (cirrhosis group) and 206 (80.5%) did not (non-cirrhosis group). The cumulative cirrhosis incidence at 5, 10, and 15 years of follow-up was 9.7%, 18.2%, and 26.4%, respectively. The HCC incidence was higher in the cirrhosis group [23/50 (46%)] than the non-cirrhosis group [8/206 (3.9%); $p < 0.0001$]. In the entire population, 71 of 82 (86.6%) patients who developed HCC had underlying cirrhosis and 11 (13.4%) did not, when HCC was detected ($p < 0.0001$).

Cumulative cirrhosis incidence according to the amino acid pattern in the core region for F0–F2 patients

The cumulative cirrhosis incidence tended to be higher in the non-DW-type group than the DW-type group (11.9% and 3.6% at 5 years, 21.5% and 10.4% at 10 years, and 29.7% and 20.7% at 15 years of follow-up, respectively; $p = 0.051$; Fig. 4).

Analysis of factors associated with cirrhosis development in F0–F2 patients

We analyzed the factors associated with cirrhosis development in patients with F0–F2 fibrosis at enrollment. Univariate analysis revealed nine parameters correlating with cirrhosis development: male gender ($p = 0.04$), older age ($p < 0.0001$), advanced fibrosis ($p < 0.0001$), absence of SVR ($p < 0.0001$), high AST level ($p < 0.0001$), high ALT level ($p = 0.01$), high AST/ALT ratio ($p = 0.001$), low platelet count ($p = 0.0009$), and high core protein level ($p = 0.02$). Multivariate analysis, including analysis of the amino acid pattern in the core region with the Cox proportional-hazards model, showed that the following three independent parameters were significantly associated with cirrhosis development: male gender ($p = 0.004$), fibrosis = F2 ($p = 0.004$), and absence of SVR ($p = 0.02$). Meanwhile, the presence of the non-DW-type pattern in the core region tended to lead to cirrhosis development (RR, 2.13; 95% CI, 0.93–4.91; $p = 0.07$).

Analysis of factors associated with mutations at core residues 70 and 91

Eighty-one patients with the DW-type pattern at core residues 70 and 91, who were at low risk for HCC, tended to be younger than the 216 patients with the non-DW-type pattern, who were at high risk for HCC (48.4 ± 11.8 years vs. 51.1 ± 11.8 years, respectively; $p = 0.08$). Separate analysis of men and women (Fig. 5) showed that the DW-type pattern was rare in women aged 60 years or above ($p = 0.02$).

Consistent with these results, HCC incidence was the same in men and women aged 60 or above (19% vs. 10% at 5 years and 32% vs. 38% at 10 years of follow-up, respectively; $p = 0.89$); however, in patients aged less than 60 years, HCC incidence was lower in women than in men (4% vs. 11% at 5 years and 15% vs. 22% at 10 years of follow-up, respectively; $p = 0.03$).

Discussion

Male gender, older age, advanced-stage fibrosis, and no IFN treatment are reported as important predictors of HCC development in chronic hepatitis C patients [4–7]. Viral factors associated with HCC development were also reported [27–29]. Several studies showed that mutations in the core protein are associated with HCC among HCV genotype 1b-infected patients, but the results varied between studies [18,30,31]. Consistent with a report by Akuta et al. [18], we showed that the presence of the non-DW-type pattern at core residues 70 and 91 is an independent risk factor for HCC development. Akuta et al. [18] studied 313 chronic hepatitis C patients who received IFN therapy (101 were excluded), and found that non-DW-type was an independent risk factor for HCC development (RR, 5.92; 95% CI, 1.58–22.2; $p = 0.008$) by using the Cox proportional-hazards model, and its correlation with HCC risk was stronger than that found in our study (RR, 1.96; 95% CI, 1.02–3.76; $p = 0.04$). We analyzed cirrhotic patients (14.7% of total population), most of whom developed HCC, and also non-cirrhotic patients, and found that the non-DW-type was still an independent risk factor for HCC development (RR, 2.90; 95% CI, 1.11–7.61; $p = 0.03$). Furthermore, we

found that the non-DW-type in patients with F0–F2 fibrosis was likely to lead to cirrhosis, diagnosed by US ($p = 0.051$). Moreover, the non-DW-type in patients with F0–F3 fibrosis was significantly associated with cirrhosis development ($p = 0.007$, data not shown). These results suggest that the non-DW-type may affect HCC development by accelerating cirrhosis development; however, prospective studies of histological findings are needed to confirm this.

It is unclear why the amino acids at residues 70 and 91 affect HCC development. The core protein cooperates with the Ras oncogene and transforms primary rat embryo fibroblasts into the tumorigenic phenotype [10]. The HCV core protein (residues 25–91) also interacts with the heterogeneous nuclear ribonucleoprotein K, which stimulates the c-myc promoter, downstream of the Wnt/beta-catenin signal [11]. Pavio et al. reported that the HCV core (residues 59–126, residues at 70 and 91 were non-wild-type) interacts with Smad3 and inhibits the TGF-beta pathway, important in apoptosis [12]. Mutations in the clustering variable regions (residues 39–76) are often seen in HCC patients [30], and mutations in the *N*-myristoylation sites (e.g., residue 91) in the core region, are associated with growth control and virus replication [31]. Delhem et al. have shown that the core protein with non-wild-type amino acids at residues 70 and 91 obtained from a HCC patient binds and activates PKR, which might cause carcinogenesis [13]. It was reported that the presence of a non-wild-type amino acid at residue 91 enhances internal initiation of HCV protein synthesis, leading to the expression of a core isoform, which may interact with viral and cellular components [32]. These results suggest that residues 70 and 91 themselves or via interactions with adjacent amino acids may be involved in HCC development; however, further studies are needed to evaluate the effect of core mutations on HCC development.

The presence of the DW-type pattern in the core region is also reportedly a predictor of the virological response to therapy with peginterferon and ribavirin [19]. With this therapy, an SVR of approximately 50% could be achieved by HCV genotype 1-infected patients having high viral load. We found the absence of an SVR and the non-DW-type pattern to be predictors of HCC development; however, the non-DW-type pattern was not a predictor of the absence of an SVR. This may be partly because we used IFN monotherapy without ribavirin, with which the SVR rate (26.5% in our study) was lower than that with peginterferon plus ribavirin [33,34]. Therefore, we believe that combination therapy, rather than IFN monotherapy, would more efficiently eradicate HCV with the DW-type pattern in the core region; however, further studies are required to test this hypothesis. Our current focus is on a prospective study to examine the association between core mutations and the outcome of combination treatment with peginterferon plus ribavirin.

Our study revealed that the DW-type pattern, associated with a low HCC risk, was rare in women aged 60 years or above. This may explain why HCC incidence in women was as high as that in men. The underlying mechanisms by which age or gender influence core-region mutations are unknown. In previous studies, a mutation at residue 70 was correlated with virological response to therapy with IFN plus ribavirin [17] and with AFP levels [35] in HCV genotype 1b-infected patients without HCC. Further follow-up studies must examine whether a mutation occurs in the wild-type amino acid.

We investigated two specific amino acid mutations in the HCV core region by direct sequencing. The HCV core sequence can be easily amplified using PCR because of its conservative nature and analysis of only two amino acid positions is timesaving; therefore, this method might be feasible for identifying predictive markers for HCC. A specific PCR method for detecting these mutations was reported [36]. Furthermore, we developed a rapid and sensitive real-time PCR method for quantitatively detecting these mutations [37]. We hope this method can be used to detect HCV sequences in case of a low viral load, and believe that it will be more useful for predicting HCC.

In conclusion, HCC risk could be predicted by studying mutations in the HCV core region, response to IFN, and host factors like age, gender, and liver fibrosis in HCV genotype 1-infected patients. These mutations might be involved in an oncogenic mechanism leading to HCC development in chronic HCV patients.

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ORIGINAL ARTICLE

Risk of Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Virus Infection

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Abstract

Objective. To determine the risk factors for the occurrence of hepatocellular carcinoma (HCC) in patients with hepatitis B virus (HBV) infection. ***Material and methods.*** A total of 620 patients who tested positive for hepatitis B surface antigen and were referred to Chiba University Hospital between February 1985 and March 2008 were included in the study and the following characteristics were analyzed: age, gender, status of hepatitis B e antigen, alanine aminotransferase level, HBV DNA level, and number of platelets (PLTs). ***Results.*** HCC was detected in 30 cases during the follow-up period (5.4 ± 5.1 years). Multivariate analysis revealed that age >40 years [compared with patients aged <40 years; odds ratio (OR) = 4.28; 95% confidence interval (CI) = 1.68–10.9] and PLT level <206,000/μl (compared with patients with a higher PLT level; OR = 8.50; 95% CI = 1.98–36.2) were predictive factors for HCC occurrence. In patients aged >40 years, the HBV DNA level (compared with <5.0 log copies/ml; OR = 4.22, 95% CI = 1.13–15.8) and PLT level (compared with patients with >196,000/μl PLTs; OR = 15.6, 95% CI = 2.06–118.3) were predictive factors for HCC occurrence. ***Conclusions.*** Advanced age and low PLT level were risk factors for HCC occurrence in patients with HBV infection. In patients aged >40 years, viral load was also a risk factor for HCC.

Key Words: *Hepatitis B virus, hepatocellular carcinoma*

Introduction

The clinical course of patients with hepatitis B virus (HBV) infection varies considerably [1]. Therefore, long-term follow-up studies of patients with HBV infection are quite complex and difficult. In most of the patients, the disease is either non-progressive or shows a slow progression and is usually accompanied by the loss of serum HBV DNA after seroconversion of hepatitis B e antigen (HBeAg) [2]. Some patients show continuous elevation of the alanine aminotransferase (ALT) level, which leads to cirrhosis [3]. HBV infection is also associated with an increased risk of developing hepatocellular carcinoma (HCC), which is one of the most common human cancers and causes of death. Although previous studies have attempted to determine factors influencing the prognosis of patients with HBV infection, the key factors remain to be identified. Recent studies have indicated that the serum level of HBV DNA correlates with the progression of liver diseases [1,4–6]. However, viral load alone cannot predict the occurrence of HCC in the future [7]. In this study, multivariate analyses of the risk factors for HCC occurrence were performed for data obtained from 620 patients with HBV infection who were referred to a single institute in Japan.

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Material and methods

Patients

This was a retrospective analysis. The study was approved by the ethical committee of Chiba University and written informed consent was obtained from all the patients. Of the hepatitis B surface antigen (HBsAg)-positive carriers ($n = 676$) who were referred to Chiba University Hospital between February 1985 and March 2008, those who tested positive for hepatitis C virus (HCV) antibody (anti-HCV) or had autoimmune liver disease and those who had another potential cause of chronic liver disease were excluded. The characteristics of the excluded HBsAg-positive carriers were as follows: anti-HCV positivity in 12, autoimmune liver disease in four and primary biliary cirrhosis in one. Five patients who had previously received lamivudine treatment were also excluded. Thirty-nine patients consulted a physician only once and were excluded from further analysis. Thus, a total of 620 patients were further analyzed. Serum samples were collected during diagnosis and stored at –20°C until analysis.

Serologic markers, HBV DNA quantitative assay, and genotyping

HBsAg, HBeAg, and anti-HBe levels were determined by enzyme-linked immunosorbent assay (ELISA; Abbott Laboratories, Chicago, IL) and anti-HCV was also measured by ELISA (Ortho Diagnostics, Tokyo, Japan). Serum HBV DNA levels were quantified by polymerase chain reaction (PCR) assay (Amplicor HBV Monitor; Roche Diagnostics, Basle, Switzerland); the linear range of this assay was 2.6–7.6 log copies (LC)/ml. The six major genotypes of HBV (A–F) were determined by EIA (HBV Genotype EIA; Institute of Immunology Co., Ltd., Tokyo, Japan). Aspartate aminotransferase (AST), ALT, and the number of platelets were determined and the aminotransferase to platelet ratio index (APRI) was calculated [8].

Statistical analysis

The baseline data are presented as mean ± SD. The difference in the values of clinical parameters between the two groups was analyzed by unpaired *t*-test, Welch's *t*-test, and chi-square test. The Cox proportional hazards model was used to identify factors predictive of HCC occurrence using the SPSS version 16.1 software package (SPSS Inc., Chicago, IL).

Results

Demographic characteristics of HCC and control patients

None of the study participants had HCC at entry. In total, 30 incident HCC cases (HCC group) occurred during the follow-up period. During the follow-up period, most of the patients were re-evaluated at least once a year for liver function and detection of HCC. Screening for detection of HCC was performed on the basis of typical findings of abdominal ultrasonography, dynamic CT, angiography, and/or MRI. For all patients suspected of having HCC by imaging analysis, the diagnosis of HCC was confirmed by pathological analysis. If the patient had HCC or was being treated with an antiviral drug (lamivudine or entecavir), we terminated the follow-up. At baseline, significant differences were observed in age, gender, status of HBeAg, ALT and HBV DNA levels, number of platelets (PLTs), and APRI between the HCC ($n = 30$) and control ($n = 590$) groups (Table I). The 590 patients in whom HCC was not detected during the follow-up period constituted the control group. The average follow-up period was 5.1 ± 4.1 and 5.4 ± 5.2 years in the HCC and control groups, respectively, and this difference was not significant.

Patients with HBV

The differences in age, sex, PLT and ALT levels, status of HBeAg, and HBV DNA level between the HCC and control groups were investigated. We defined threshold levels as age 40 years, HBV DNA 5.3 LC/ml, ALT 72.9 IU/l, and PLTs 206,000/μl according to the average data of all patients. Univariate analysis revealed that age, number of PLTs, and HBV DNA level at baseline were predictive factors for HCC occurrence. Multivariate analysis revealed that age >40 years [compared with patients aged <40 years; odds ratio (OR) = 4.28; 95% confidence interval (CI) = 1.68–10.9] and PLT level <206,000/μl (compared with patients with a higher PLT level; OR = 8.50, 95% CI = 1.98–36.2) were predictive factors for HCC occurrence (Table II). Thus, these analyses revealed that age and PLT level were the most important factors influencing future occurrence of HCC. Kaplan–Meier curves were constructed for age ($P < 0.0001$; log-rank test; Figure 1a), PLT level ($P < 0.0001$; log-rank test; Figure 1b), and HBV DNA (P = NS; log-rank test; Figure 1c). Next, we categorized the HBV patients into two subgroups according to the thresholds of age and PLT level based on the average data, and performed further analysis. Because there was only one HCC patient aged <40 years and

Table I. Characteristics of study subjects and their association with HCC.

Parameter	Group			*P*
	Total	HCC	Controls	
No. of patients	620	30	590	
Gender; *n* (%)				<0.001[a]
Male	364 (59)	20 (67)	344 (58)	
Female	256 (41)	10 (33)	246 (42)	
Age (years); mean ± SD	40.0 ± 14.2	50.0 ± 11.6	40.0 ± 14.2	<0.001[b]
HBeAg status; *n* (%)				<0.001[a]
Positive	269 (43)	17 (57)	252 (43)	
Negative	351 (57)	13 (43)	338 (57)	
HBV DNA (LC/mL); mean ± SD	5.3 ± 2.0	6.4 ± 1.3	5.3 ± 2.0	0.002[b]
ALT (IU/l); mean ± SD	72.9 ± 89.3	105.0 ± 129.3	71.0 ± 86.6	0.041[c]
PLTs (/μl); mean ± SD	206,000 ± 66,000	130,000 ± 51,160	210,000 ± 64,410	<0.001[c]
APRI > 0.5; *n* (%)	294 (47.4)	27 (90)	267 (45.3)	<0.001[a]
Interval between two consecutive visits (years); mean ± SD	5.4 ± 5.1	5.1 ± 4.1	5.4 ± 5.2	NS[c]
Genotype A/B/C/D/not determined; *n*	7/38/333/0/242	1/0/24/0/5	6/38/309/0/237	NS[a]

[a]Chi-square test.
[b]Welch's *t*-test.
[c]Unpaired *t*-test.

only two cases had a PLT level > 206,000/μl, we did not analyze these groups.

Analysis of the subgroup of HBV patients aged > 40 years

HCC was detected in 29 patients in the group aged > 40 years (n = 372). Significant differences were observed in the status of HBeAg, HBV DNA, and PLT levels at baseline between the HCC (n = 29) and control groups (n = 343). The average follow-up period was 5.1 ± 4.1 and 5.0 ± 4.7 years in the HCC and control groups, respectively, and this difference was not significant. We defined thresholds as age 49 years, HBV DNA 5.0 LC/ml, ALT 66.0 IU/l, and PLTs 196,000/μl, according to the average data for the patients aged > 40 years. The risk factors for HCC occurrence in patients aged > 40 years were analyzed by Cox regression analysis. Univariate analysis revealed that ALT, PLT, and HBV DNA levels at baseline were predictive factors for HCC occurrence. Multivariate analysis revealed that the HBV DNA

Table II. Multivariate analysis of risk factors associated with HCC in patients with HBV infection.

Risk factor	All patients[a]		Patients aged > 40 years[b]		Patients with PLTs < 206,000 /μl[c]	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Age	4.28 (1.68–10.9)	0.002	2.16 (0.88–5.29)	NS	1.75 (0.71–4.34)	NS
Male gender	1.48 (0.67–3.26)	NS	2.25 (0.86–5.90)	NS	1.43 (0.61–3.35)	NS
HBeAg-positive	1.34 (0.59–3.06)	NS	0.98 (0.41–2.33)	NS	1.06 (0.45–2.51)	NS
HBV-DNA	1.59 (0.62–4.13)	NS	4.22 (1.13–15.8)	0.032	1.20 (0.49–2.94)	NS
ALT	0.86 (0.40–1.87)	NS	1.44 (0.61–3.44)	NS	0.923 (0.40–2.11)	NS
PLTs	8.50 (1.98–36.2)	0.004	15.6 (2.06–118.3)	0.008	4.49 (1.62–12.5)	0.004

[a]The thresholds of age, HBV-DNA, ALT, and PLTs were defined as 40 years, 5.3 LC/ml, 72.9 IU/l, and 206,000 /μl, respectively.
[b]The thresholds of age, HBV-DNA, ALT, and PLTs were defined as 49 years, 5.0 LC /ml, 66.0 IU/l, and 196,000 /μl, respectively.
[c]The thresholds of age, HBV-DNA, ALT, and PLTs were defined as 42 years, 5.8 LC /ml, 84 IU/l, and 159,000 /μl, respectively.
HR = hazard ratio.

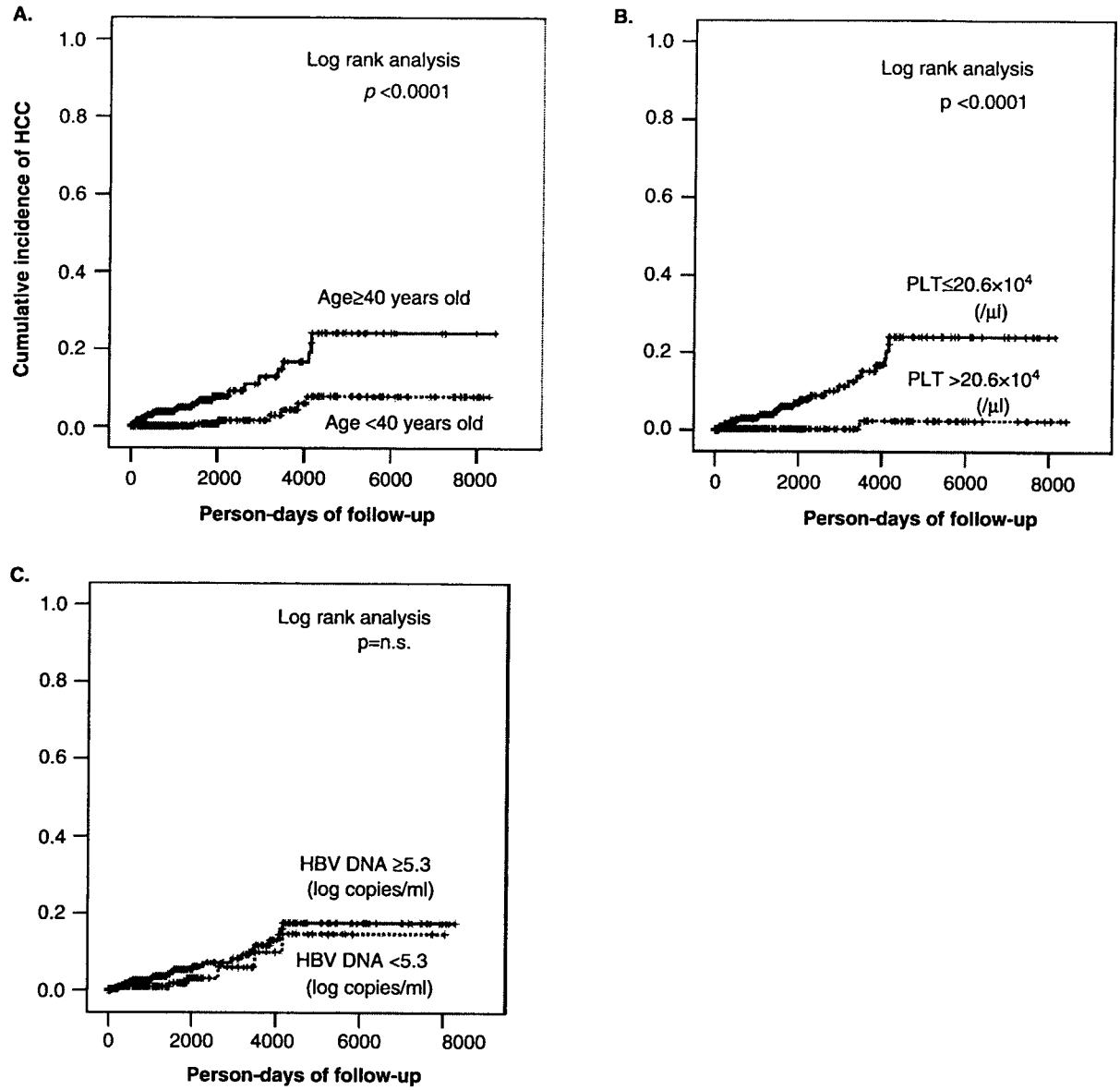

Figure 1. Cumulative occurrence of HCC based on (a) number of PLTs, (b) age, and (c) HBV DNA level. Thresholds for age, number of PLTs, and HBV DNA level were defined according to the average data for all patients. Dotted lines indicate the control group (high number of PLTs, younger age, and low HBV DNA level).

level (compared with < 5.0 LC/ml; OR = 4.22; 95% CI = 1.13–15.8) and PLT level (compared with > 196,000/μl; OR = 15.6; 95% CI = 2.06–118.3) were predictive factors for HCC occurrence (Table II). Kaplan–Meier curves were constructed for HBV DNA ($P = 0.001$; log-rank test; Figure 2).

Analysis of the subgroup of HBV patients with PLTs < 206,000/μl

HCC was detected in 28 patients in the group with PLTs < 206,000/μl ($n = 329$). The risk factors for HCC occurrence in the group with < 206,000/μl PLTs were analyzed by Cox regression analysis. Univariate analysis revealed that age and PLT level at baseline were predictive factors for HCC occurrence. Multivariate analysis revealed that PLT level (compared with patients with > 159,000/μl; OR = 4.49; 95% CI = 1.62–12.5) was the only predictive factor for HCC occurrence (Table II).

Discussion

In Japan, HBV infection is one of the most important factors determining HCC occurrence [9]. Moreover, HCC is one of the most important determinants for

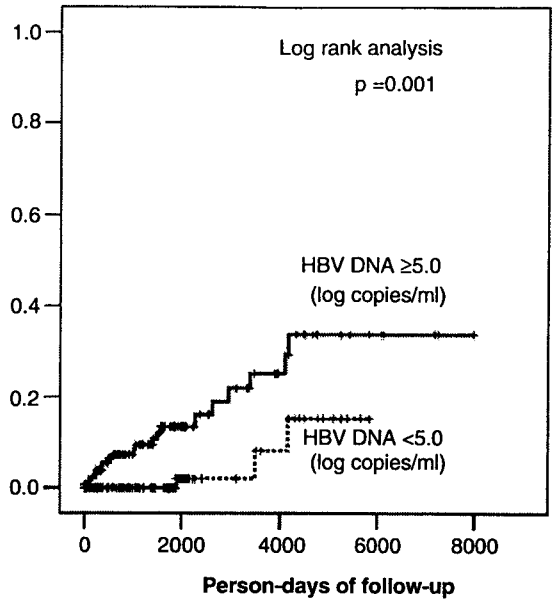

Figure 2. Cumulative occurrence of HCC based on the HBV DNA level in patients aged > 40 years. The threshold for the HBV DNA level was defined according to the average data for the patients aged > 40 years. A significant difference was observed by log-rank test. The dotted line indicates the control group (low HBV DNA level).

the prognosis of patients with HBV infection. In previous studies, factors associated with an increased risk of HCC among people with chronic HBV infection included demographic characteristics, lifestyle, and environmental, viral and clinical factors. Among these, male gender, older age, HBV genotype, cirrhosis, elevated ALT, and high viral load were found to be factors associated with HCC [6,10–19]. We focused on clinical factors which may be tested easily and for which tests are available all over the world. This report clarifies the relative risk for HCC in all patients with HBV who were referred to a single institute in Japan and provides important information for physicians.

In this study, the relative risk of HCC was found to be increased to 4.28 (95% CI 1.68–10.9) times higher for patients aged > 40 years compared with those aged < 40 years. In addition, a low PLT level, which indicates advanced fibrosis in the liver, including cirrhosis, was a risk factor for HCC: the relative risk was found to be increased to 8.50 (95% CI 1.98–36.2) times higher for patients with a PLT level < 206,000/μl compared with higher levels. The HBV DNA level was not selected as a risk factor for HCC occurrence in all patients with HBV infection by multivariate analysis. Previous follow-up studies have shown that viral load is an important and independent factor for HCC occurrence [4,5,20]. However, in the present study, although various thresholds of HBV DNA level were used for analysis, none of the thresholds showed statistical significance in multivariate analysis (data not shown). In contrast, the analysis intended for patients aged > 40 years revealed that high HBV viral load was added as a risk factor for HCC. By changing the threshold of HBV DNA from 4.5 to 5.3 LC/ml in 0.1-log increments, 5.0 or 5.1 LC/ml were found to be the best (data not shown); therefore we designated the threshold of HBV DNA level as > 5.0 LC/ml. In our study, HBV carriers aged > 40 years with HBV DNA levels > 5.0 LC/ml had a 4.22-times higher risk of HCC compared to HBV carriers with lower viral loads. In previous studies in Japan regarding predictive factors for HCC, Ohata et al. [5] reported that age, HBV DNA, and staging of fibrosis were the important factors, while Murata et al. [21] reported that the number of PLTs was the only factor after HBeAg seroconversion. On the other hand, in an analysis of patients with liver cirrhosis in Japan, levels of HBV DNA and/or ALT were the predictive factors for HCC [12,19]. Taken together with the present study, these reports suggest that the HBV DNA level may not be an absolute factor for predicting HCC in the analysis, irrespective of the age of the patients and the number of PLTs, but that in patients with advanced age or low numbers of PLTs, indicating advanced fibrosis of the liver, HBV DNA could be a predictive factor for the occurrence of HCC. The PLT level negatively reflects the extent of liver fibrosis [22], therefore it is very difficult to achieve an improvement in liver fibrosis and to recover the PLT level concomitantly, but a high viral load can be lowered by antiviral drug treatment. Therefore, in patients aged > 40 years, lowering the viral load using an antiviral drug might be an important way to avoid the occurrence of HCC but, in younger patients, lowering the HBV DNA level may not result in direct inhibition of HCC occurrence, although the activity of hepatitis could be suppressed.

The decrease in the number of PLTs in patients with liver disease reflects advanced fibrosis of the liver, which is strongly related to HCC occurrence. In fact, the patients in the HCC group of our study were suggested to show advanced fibrosis because they had higher values of APRI than the controls. In addition to being a marker of liver fibrosis, the influence of PLTs on cytotoxic T lymphocytes (CTLs) has been studied with keen interest. Chronic HBV infection is characterized by an inefficient CTL response, which often results in continuous destruction of hepatocytes. A recent study indicated that PLTs are required for virus-specific CTLs to accumulate within the liver and perform pathogenetic and/or antiviral roles [23]. In our study, low PLT number was a strong risk factor for HCC in all the HBV carriers, irrespective of age or PLT number at baseline. Especially in the HBV

carriers aged >40 years, low PLT number has the strongest association with HCC occurrence. Therefore, older HBV carriers with low PLT levels should be followed closely because of a high possibility of HCC occurrence, as for HCV carriers with low PLT levels [24].

The presence of HBeAg is often associated with active liver disease, whereas HBeAg seroconversion often coincides with loss of HBV DNA in serum, normalization of the ALT level, and clinical remission [25]. Spontaneous HBeAg seroconversion confers a good long-term outcome on most patients. In this study, the status of HBeAg at baseline differed significantly between the HCC and control groups; however, the status of HBeAg was not identified by univariate analysis as a predictive factor for HCC occurrence. From these results, we speculated that the HBe protein was not the direct precursor of HCC, although the HBe antigen status often reflects the replication of HBV DNA.

In this study, we evaluated parameters for predicting HCC only at first admission. A previous study reported that changes in ALT or HBV DNA levels during the follow-up period were important for predicting advanced liver disease and HCC [26]. We need to evaluate the importance of following changes in these parameters.

There was only one HCC patient aged <40 years. This patient was male and was followed up from the age of 27 years; his ALT, HBV DNA, and PLT levels and the status of HBeAg at baseline were 34 IU/l, 7.7 LC/ml, 203,000/μl, and positive, respectively. It was difficult to predict the occurrence of HCC in this case only on the basis of the risk factors for HCC indicated in this study. Hence, we need to find an adequate risk factor to predict HCC in such a case.

In conclusion, advanced age and low PLT level were the risk factors for HCC in patients with HBV infection, irrespective of the PLT level at baseline. In patients aged >40 years, viral load was added as a risk factor for HCC.

Declaration of interests: The authors indicated no potential conflict of interest.

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