Table VIII. Frequency alleles of IL-18 gene-promoter polymorphism in inactive HBV carriers and CPLD patients

Loci	Inactive HBV carrier (n = 86)	CPLD (n = 322)	P value
Locus -607			
C	31 (36.0)	148 (46.0)	0.128
A	55 (64.0)	174 (54.0)	
Locus -137			
G	71 (82.6)	295 (91.6)	0.024
C	15 (17.4)	27 (8.4)	

Table IX. The distribution of IL-18 genotype and fibrosis

	F0–F2 (n = 21)		F3–F2 (n = 36)		OR (95% CI)	P value
	n	%	n	%		
Genotype -607						
A/A	6	28.6	8	22.2	0.71 (.021–2.44)	0.591
A/C	13	61.9	21	58.3	0.86 (0.29–2.59)	0.791
C/C	2	9.5	7	19.4	2.29 (0.43–12.23)	0.461
Genotype -137						
G/G	17	81.0	30	83.3	1.10 (0.29–4.76)	>0.999
G/C	4	19.0	5	14.9	0.69 (0.16–2.90)	0.712
C/C	0	0.0	1	2.8	NA	>0.999

NOTES: P-value: The Fisher exact test.
ABBREVIATIONS: NA, not available.

cytokine genes.[5] Furthermore, cytokine gene polymorphisms have been shown to be associated with the disease progression of HBV infection.[4] It is crucial to identify genetic factors that determine the outcome of HBV infection, because these factors may reveal new therapeutic opportunities for patients with chronic HBV infection.

IL-18, which is a proinflammatory cytokine that belongs to the IL-1 family, induces IFN-γ production in T cells and natural killer cells, playing an important role in the Th_1 response.[7] However, the role of IL-18 in regulation of HBV infection has yet to be fully defined. IL-18 exerts a synergistic effect on IFN-γ production and induces antiviral activities.[18,19] It was also reported that IL-18 inhibited HBV replication in livers of HBV transgenic mice.[20] In addition, IL-18 is also known to induce the production of Th_2 cytokine, such as IL-4 and IL-13.[21] The dual role of IL-18 in Th_1 and Th_2 cytokine production could be implicated in the immune response against HBV infection.

In the current study, we compared the distributions of IL-18 gene promoter polymorphisms among Japanese HBV-infected patients with different clinical outcomes. Our results demonstrated that the frequencies of the -607 AA genotype and the -137 C allele were significantly higher in the inactive HBV carriers compared with those in patients with CPLD. The results of the current study suggest that the -607 AA genotype and the -137 C allele have a protective effect on the disease progression of HBV-related liver disease. Although the -607 A/A genotype was associated with a reduced risk of the progression of HBV infection (OR, 0.41), the -607 A/C genotype was associated with an increased risk of the progression of HBV infection (OR, 2.07). The mechanisms for the differential effects of these 2 genotypes on the development of HBV infection are not clear in this study. It was reported that these 2 polymorphisms -137 G/C and -607 C/A were in strong linkage disequilibrium.[22] In our study, there was a large difference in -137 C allele frequencies between the -607 AA genotype and the -607 AC or CC genotypes (-607 AA:21.7% vs -607 AC/CC:7.1%). It is possible that the protective effect of the -607 AA genotype on the development of HBV infection are attributable to the other loci, which are in linkage with -607 AA genotype, such as -137 C allele.

The presence of the C allele at position -607 (C/C+C/A) has been shown to be associated with a higher risk of cirrhosis and HCC in HBV-infected patients.[23] However, we did not find a significant association of the -607 genotype and severe fibrosis and HCC occurrence in our HBV-infected patients.

The current findings lead to address the question as to how IL-18 polymorphisms are related to the progression of HBV-related liver disease. Giedraitis et al[9] demonstrated that the allele C at -137 has been shown experimentally to disrupt the confirmed H4TF-binding site, whereas nucleotide substitution at -607 (C→A) may disrupt a the potential cyclic-adenosine-monophosphate–responsive element-binding site.[9] Furthermore, in an IL-18 promoter transcription activity assay, it was demonstrated that the presence of both A and C alleles at positions -607 and -137 in the same haplotype is associated with low promoter activity.[9] Our results may not be in good accordance with these findings of Giedratis et al.[9] Mechanisms underlying the relationship between the IL-18 gene promoter polymorphisms and the outcome of HBV infection are not clear in our study. Zhang et al reported that the carriage of the allele C at position -137 in the promoter of IL-18 gene may play a protective role in the development of HBV infection, and the AA genotype at position -607 may be associated with HBV-DNA replication.[24] Our results are concordant with these previous findings that IL-18 promoter polymorphisms could influence the outcome of HBV infection. More recently, Hirankarn et al[25] demonstrated an association between -607 A/A polymorphism and the susceptibility of chronic HBV infection. However, the state of HBV infection and the degree of liver damage in their studied population were not described. To establish firmly the relationship between IL-18 gene

promoter polymorphisms and the risk of the progression of HBV infection, more large-scale studies are required that include individuals of other ethnicities.

In conclusion, we have attempted to elucidate the role of genetic polymorphisms of IL-18 gene in the outcome of HBV infection. Our data suggest that the polymorphisms at the IL-18 gene promoter region (-607 and -137) may affect the development and progression of HBV-related liver disease.

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Gain of *GRHL2* is associated with early recurrence of hepatocellular carcinoma☆

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Background/Aims: The aim of this study is to identify genomic changes that might be implicated in hepatocellular carcinoma (HCC) progression, and evaluate the associations with clinico-pathological features.

Methods: The genomic DNA of 17 hepatoma cell lines was analyzed using Affymetrix GeneChip Human Mapping 50 K high-density oligonucleotide arrays. We selected representative genes from recurrent amplified regions and measured the copy number of these genes in 70 HCC clinical samples.

Results: We found 10 recurrent high-grade gain regions spanning less than 3 Mb in at least two hepatoma cell lines, and selected 10 representative genes. The copy number was almost normal in non-cancerous tissue and frequently amplified in Edmondson grade II or III HCC compared to Edmondson grade I HCC. Gain of _TAX1BP1_ in 7p15.2-1 was associated with larger tumor size and positivity of HCV antibody, and gain of _CCND1_ in 11q13.2-3 was associated with larger tumor size by multivariate analysis. Furthermore, a gain of _GRHL2_ in 8q22.3 was associated with early recurrence of HCC, controlling for clinical parameters. Decreased _GRHL2_ expression by RNA interference inhibits the growth of hepatoma cells, suggesting its association with cell proliferation.

Conclusions: A gain of _GRHL2_ might be a predictive marker for HCC recurrence.
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Keywords: **Copy number alteration; Loss of heterozygosity; High-density single nucleotide polymorphism array; Genetic marker; Recurrence**

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Abbreviations: CGH, comparative genomic hybridization; CNAG, copy number analyzer for Affymetrix GeneChip Mapping 100 K arrays; FACS, fluorescence activated cell sorting; FFPE, formalin-fixed paraffin-embedded; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV Ab, hepatitis C virus antibody; HMM, hidden Markov model; LOH, loss of heterozygosity; SNP, single nucleotide polymorphism; TSG, tumor suppressor genes.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies worldwide, and its incidence continues to increase in the United States and Europe due to the spread of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection [1]. The treatment of HCC is limited because of underlying cirrhosis and a high rate of recurrence; the cumulative 5-year survival rate is 53.4% with hepatic resection, 42.0% with local ablation therapy, and 22.6% with transcatheter arterial embolization in Japan [2]. Radio-frequency ablation therapy has been recently introduced and has improved the survival rate. Nevertheless, even in small HCC, the 4-year survival rate is only 74% [3]. The recurrence and prognosis of HCC is highly dependent on tumor extension and liver function. Prognostic scoring systems, such as CLIP score [4], BCLC score [5], JIS score [6] and Tokyo score [7] have been widely used to evaluate the prognosis of HCC patients. However, each system cannot always be sufficient for prediction of recurrence and prognosis, therefore identification of molecular markers that complement clinical information for recurrence could help attain a better follow-up of patients.

The genesis and progress of HCC are considered to be due to the accumulation of genetic alterations, including point mutations in oncogenes or tumor suppressor genes (TSGs). At the same time, copy number aberration or loss of heterozygosity (LOH) in the chromosome can lead to the activation of oncogenes and inactivation of TSGs, resulting in carcinogenesis [8,9]. The regions of copy number gain and loss are supposed to contain putative oncogenes or TSGs respectively, and some reports revealed that they are associated with clinico-pathological features or recurrence of HCC [10,11]. However, other reports failed to show any correlation between them [12–14], and predictive potential of allelic imbalance for clinical parameters is still uncertain.

Recently, single nucleotide polymorphism (SNP) arrays have been applied to detect SNPs as well as DNA copy number changes at the genome level [15,16]. SNP arrays allow the genome-wide measurement of gene copy number changes and LOH simultaneously with high accuracy for several types of cancers, including HCC [17]. In this report, 36 HCCs were analyzed using Affymetrix GeneChip Human Mapping 10 K high-density oligonucleotide arrays. However, association between allelic imbalance and clinico-pathological parameters was not shown.

To find the molecular markers that associate with clinical parameters, we analyzed the genome of 17 hepatoma cell lines using Affymetrix GeneChip Human Mapping 50 K high-density oligonucleotide arrays, and evaluated the association between allelic imbalance and clinical parameters. In this study, we focused on the regions that contain putative oncogenes and analyzed a copy number of representative 10 genes (derived from 10 recurrent amplified regions of high-grade gain spanning less than 3 Mb in at least two cell lines) in HCC clinical samples. An analysis of the gain of genes and clinico-pathological features revealed that the gain of some chromosomal regions is associated with certain clinical characters such as tumor size and HCV infection. Moreover, the gain of *GRHL2* in 8q22.3 was associated with recurrence-free survival, suggesting that gain of *GRHL2* might be a novel genetic marker for predicting HCC recurrence.

2. Materials and methods

2.1. Cell lines

We used 17 cell lines derived from human hepatomas; PLC/PRF/5, HuH-1, HuH-6, HuH-7, HepG2, Hep3B, HLE, HLF, SK-Hep1, JHH-1, JHH-4, JHH-7, SNU-398, SNU-449, SSP-25, HT17 and RBE (Table 1). HuH-1, HuH-7, HLE, HLF, JHH-1, JHH-4, and JHH-7 were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). HT17 was obtained from the Cell Resource Center for Biomedical Research (Sendai, Japan). PLC/PRF/5, HuH-6, HepG2, SSP-25, and RBE were obtained from RIKEN Bioresource Center (Tsukuba, Japan), and the remainder from the American Type Culture Collection (Manassas, VA, USA). The cells were grown under the recommended culture conditions. Genomic DNA was extracted using the Puregene DNA Isolation Kit (Gentra, Minneapolis, MN, USA) according to the manufacturer's instructions. Ploidities of hepatoma cell lines were measured with fluorescence activated cell sorting (FACS) and provided an estimate of the DNA content of each cell line.

2.2. Primary tumor tissues

To analyze the copy number of clinical samples, we used 30 pairs of frozen surgical HCC specimens and 40 pairs of formalin-fixed, paraffin-embedded (FFPE) HCC needle biopsy specimens and their corresponding non-tumor liver tissue specimens. They were obtained at the time of operation or tumor ablation therapy. FFPE specimens were obtained by ultrasound-guided needle biopsy with a 20-gauge needle (Bard Monopty; C.R. Bard, Covington, GA, USA). All patients gave informed consent before their participation, and the Ethics Committee of the University of Tokyo and Chiba University approved these studies.

Table 1
List of hepatoma cell lines

Cell line	Age	Sex	Type
PLC/PRF/5	24	M	Hepatocellular carcinoma
HuH-1	53	M	Hepatocellular carcinoma
HuH-6	1	M	Hepatoblastoma
HuH-7	57	M	Hepatocellular carcinoma
HepG2	15	M	Hepatocellular carcinoma
Hep3B	8	M	Hepatocellular carcinoma
HLE	68	M	Hepatocellular carcinoma
HLF	68	M	Hepatocellular carcinoma
SK-Hep1	52	M	Cholangiocellular carcinoma
JHH-1	50	M	Hepatocellular carcinoma
JHH-4	51	M	Hepatocellular carcinoma
JHH-7	53	M	Hepatocellular carcinoma
SNU-398	42	M	Hepatocellular carcinoma
SNU-449	52	M	Hepatocellular carcinoma
SSP-25	64	F	Cholangiocellular carcinoma
HT17	32	M	Hepatocellular carcinoma
RBE	64	F	Cholangiocellular carcinoma

Microdissections of FFPE samples were performed as previously described [18]. Samples were cut into serial sections 10 μm thick, and the sections were stained with hematoxylin for histological visualization. Microdissection was performed on all FFPE samples using the Application Solutions Laser Microdissection System (Leica, Tokyo, Japan) to ensure that only tumor cells or non-tumor cells were dissected. Genomic DNA from FFPE tissue and frozen surgical tissue was extracted using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.3. Single nucleotide polymorphism array and data analysis

Array experiments were conducted according to the standard protocols for Affymetrix GeneChip Human Mapping 50 K arrays (Affymetrix, Santa Clara, CA, USA) as previously described [16]. All examples of copy number and LOH analysis using Affymetrix GeneChip Human Mapping 50 K arrays in the cell lines were performed with Copy Number Analyzer for Affymetrix GeneChip Mapping 100 K arrays (CNAG) software using the hidden Markov model (HMM) and a maximum likelihood method, respectively [16,19]. CNAG software is available at http://www.genome.umin.jp. Mapping information for SNP locations and cytogenetic bands were based on Affymetrix and University of California, Santa Cruz, hg 16 (http://genome.ucsc.edu/) data.

2.4. Quantitative real-time PCR

Quantitative real-time PCR was performed on an ABI PRISM 7000 Sequence Detection System using the SYBR GREEN PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The standard curve method was used to calculate the target gene copy number in the hepatoma cell lines and primary HCC normalized to LINE-1, a repetitive element for which copy numbers per haploid genome are similar among all normal and neoplastic human cells [15]. The relative target copy number level was also normalized to normal human genomic DNA as a calibrator. Primer sequences are summarized in Supplemental Table 1.

2.5. Statistical analysis

We used the following variables obtained from the surgical procedure or ablation therapy in analyzing associations with clinico-pathological parameters: age, sex, positivity for viral markers (hepatitis B virus surface antigen and anti-hepatitis C virus antibody), liver function as classified by Child-Pugh scores (5–6 points, class A; 7–8 points, class B; and 9–12 points, class C; based on albumin, bilirubin, prothrombin activity, and the presence of ascites or hepatic encephalopathy), and tumor factors, including size, number of nodules, tumor histology, and background histology. Continuous variables were transformed into two subset categories.

Comparisons of different groups were performed using Fisher's exact test and logistic regression. A cumulative recurrence-free survival curve was plotted using the Kaplan–Meier method. Recurrence time was defined as the interval between ablation and the detection of HCC recurrence. The difference between subgroups was tested by the log-rank test. The risk for recurrence was assessed with a Cox proportional hazard regression model, controlling for clinical parameters including tumor histology and size at the time of treatment and number of recurrence. All statistical analyses were performed with JMP 6.0.3 (SAS Institute, Cary, NC, USA). Values of $p < 0.05$ were considered statistically significant.

2.6. Construction and transfection of the vector for GRHL2 RNA interference

Construction and transfection of the vectors for *GRHL2* RNA interference was performed as previously described [20]. Briefly, a plasmid expressing a double-strand small interfering RNA against the *GRHL2* gene was generated from the pcPUR+U6i cassette vector (pcPUR). The sequence targeting the *GRHL2* was GATGAAG CCTGGAAGTCAT (pcPUR/siGRHL2-1) and GGCAGAAGATG AATTGAAG (pcPUR/siGRHL2-2). For transfection, the cells were seeded onto 10-cm dishes, and pcPUR/siGRHL2-1, pcPUR/siGRHL2-2, or control pcPUR vector was added to each dish 24 h later. The transfected cells were cultured for 24 h in the appropriate medium containing 2 μg/mL puromycin (Wako, Osaka, Japan) followed by an additional 72 h in medium without puromycin. Transfected cells were used for the preparation of total RNA for reverse transcription-PCR assay and proliferation assays.

2.7. Reverse transcription-PCR and quantitative reverse transcription-PCR analysis

Total RNA was extracted from cultured cells using ISOGEN reagent (NipponGene, Tokyo, Japan), and the extracted RNA was reverse transcribed and amplified by reverse transcription-PCR using the QuantiTect Reverse Transcription Kit (Qiagen). The quantitative reverse transcription-PCR was performed as described in Section 2.4. Each sample was examined in triplicate, and the amounts of the PCR products produced were normalized with respect to the GAPDH internal control. The following primer pairs were used: GAPDH, 5′-TGGGATTTCCATTGATGACAAG-3′ and 5′-CC ACCCATGGC AAATTCC-3′; *GRHL2*, 5′-GGAAATC TAGCCCTGGGTTTG-3′ and 5′-TCAGGGAGGAAC GCACTGA-3′.

2.8. Cell proliferation assay

The cells were seeded onto six-well plates at a density of 2×10^4 cells per well. After removal of puromycin, the number of viable cells was determined in triplicate wells at 48 and 72 h using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma–Aldrich, St. Louis, MO, USA).

3. Results

3.1. Overview of copy number alterations in hepatoma cell lines

We analyzed 17 liver cancer cell lines, 13 hepatocellular carcinomas, 3 cholangiocellular carcinomas, and 1 hepatoblastoma (Table 1) using Genechip and CNAG. The absolute copy number was measured and estimated with FACS. The DNA of 17 human hepatoma cell lines was hybridized to SNP arrays containing over 50,000 mapped SNP loci. CNAG, a copy number detection algorithm, allowed the assessment of copy number and the identification of genomic gains and deletions by HMM.

Fig. 1 shows an overview of the amplification and deletion of 17 cell lines. Overall, the genome of hepatoma cell lines is broadly amplified due to their polyploidity, and the gain of a chromosome is dominant compared to the loss of a chromosome.

3.2. Overview of LOH status in hepatoma cell lines

The LOH regions across the entire genome of all 17 cell lines were suggested by SNP array analysis using a maximum likelihood method (Fig. 2). To greater or lesser degrees, LOH was predicted in all of the chromosomes. The LOHs of 8p, 9p, 13q, and 17p were detected at high frequencies.

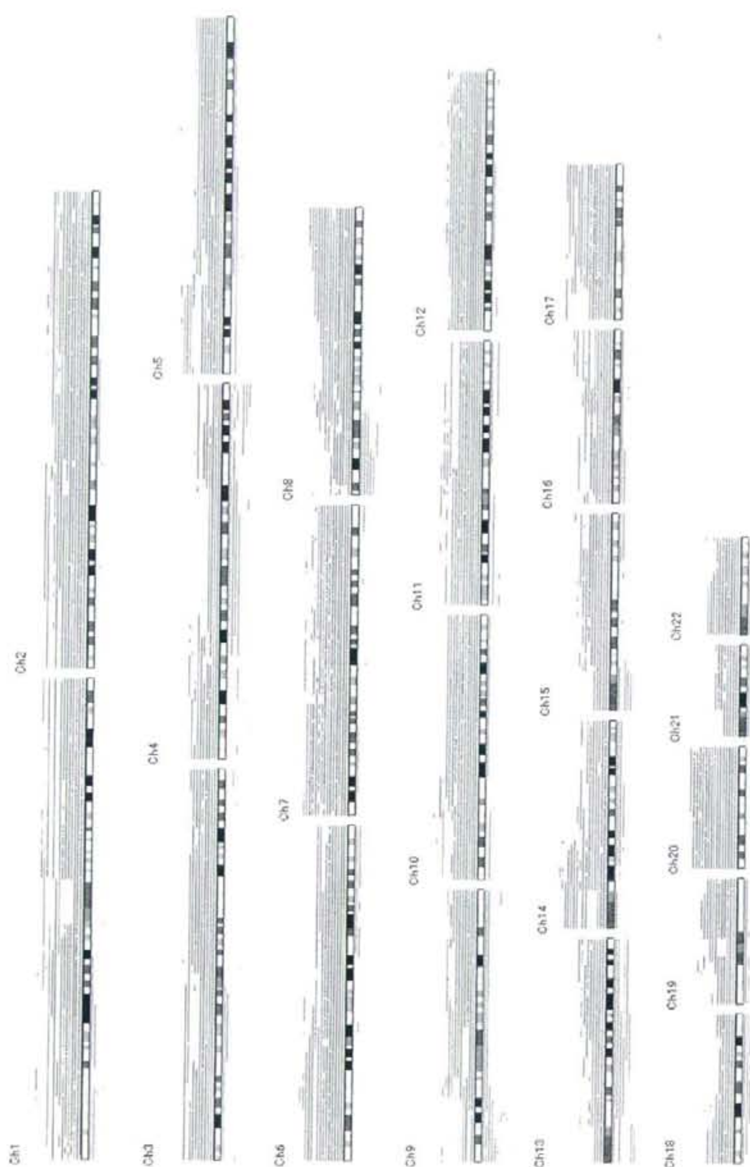

Fig. 1. Overview of copy number alterations in 17 liver cancer cell lines. The green bar shows the region of copy number gain ($n \geqslant 3$), the red bar shows the region of copy number amplification ($n \geqslant 5$), and the blue bar shows the region of copy number deletion ($n \leqslant 1$) in each cell line.

3.3. High-grade gain in hepatoma cell lines

Regions of high-grade gain were defined as segments of at least two SNP loci with an estimated copy number of five or more by HMM (Fig. 1). To find small regions useful for localizing novel putative oncogenes, we looked at the recurrent regions of high-grade gain spanning <3 Mb in at least two cell lines (Table 2). In total,

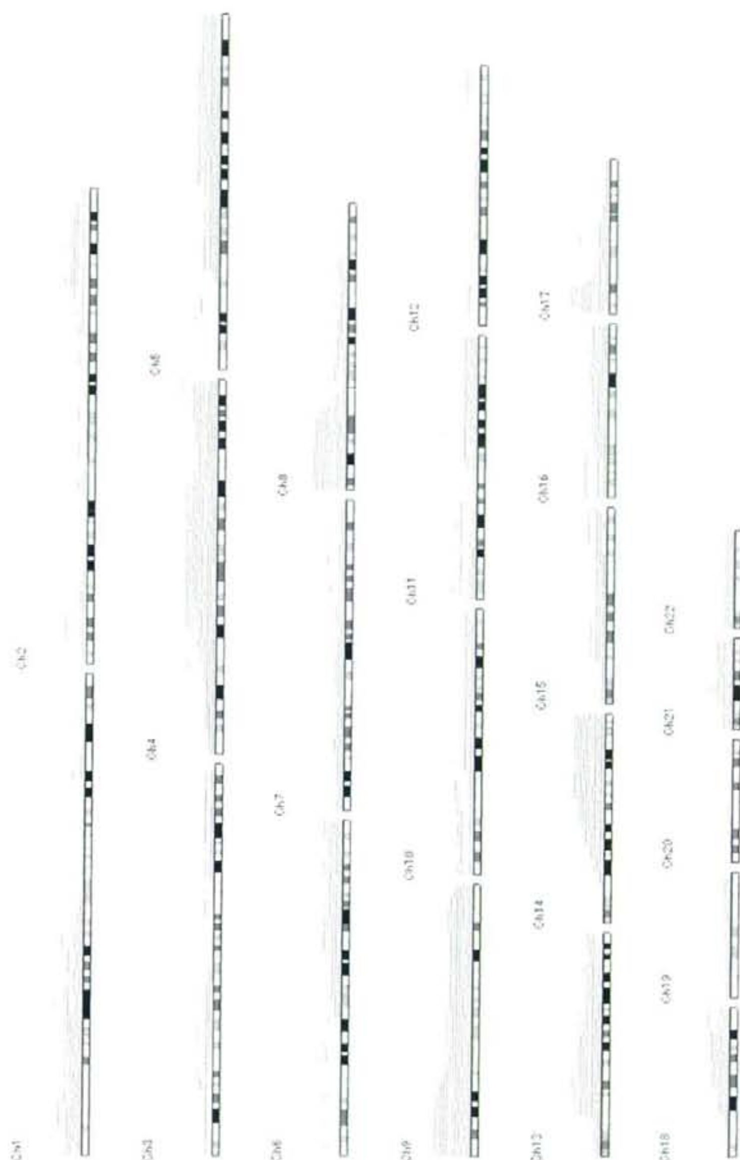

Fig. 2. Overview of the LOH regions in 17 liver cancer cell lines. The bar indicates the LOH region inferred from the GeneChip Human Mapping 50 K arrays and CNAG in each cell line.

10 loci were detected as candidate regions for high-grade gain in the genome. To confirm the high-grade gain, we selected genes that were contained in the regions of high-grade gain and subjected them to quantitative real-time PCR. We chose 10 genes previously reported to be cancer-related genes in several types of cancer, *PTCH2* [21], *CCND1* [22,23], *PPFIBP1* [24], *PRKCM* [25], *MAPK7* [26], or genes poorly characterized for their function,

Table 2
Common amplification regions in 17 hepatoma cell lines

No.	Cytoband[a]	Start (kb)[a]	Stop (kb)[a]	Length (kb)[b]	Total number of genes	Representative gene[c]	Cell lines	Copy number quantified by real-time PCR
1	1p34.1	44,197	44,771	574–901	11	*PTCH2*	HuH-6 JHH-1	8.50 ± 0.54 14.6 ± 0.44
2	1p22.1-21.3	93,791	94,322	531–712	5	*ABCA4*	HuH-6 JHH-1	5.36 ± 0.39 7.11 ± 0.84
3	7p15.2-1	231,513	233,161	1648–1861	3	*TAX1BP1*	HuH-6 JHH-1	7.94 ± 0.81 17.1 ± 1.57
4	8q22.3	102,284	102,721	437–477	3	*GRHL2*	HuH-6 JHH-1	5.26 ± 1.30 2.49 ± 0.40
5	11q13.2-3	68,089	69,728	1639–3064	15	*CCND1*	Hep3B JHH-7 HT17	3.24 ± 0.12 23.6 ± 2.59 28.6 ± 1.04
6	12p11.23-22	27,173	27,960	787–916	5	*PPFIBP1*	HuH-6 JHH-1	9.50 ± 1.06 9.02 ± 0.59
7	12p12.3	19,118	19,295	177–378	1	*PLEKHA5*	HuH-6 SNU-449 SSP-25	8.77 ± 0.39 2.70 ± 0.17 3.62 ± 0.32
8	14q12	28,090	30,519	2429–2558	15	*PRKCM*	HuH-7 HT17	11.7 ± 1.14 13.3 ± 1.54
9	17p11.2	19,432	19,561	129–805	3	*MAPK7*	JHH-7 SNU-449	12.9 ± 0.56 33.7 ± 3.63
10	22q12.2	28,672	28,908	236–1250	3	*MTMR3*	JHH-1 RBE	12.8 ± 0.78 6.66 ± 0.19

Note: The data include the regions with known genes that were amplified ($n \geq 5$) in at least two cell lines, and the size was not >3 Mb.
[a] Based on the UCSC Genome Browser July 2003 (hg16) Assembly.
[b] The minimal size range is the distance between the ends of consecutive SNPs that meet the criteria. The maximal size range is the distance between the SNPs that flank the regions.
[c] The representative genes were verified by real-time PCR. It does not imply that the indicated gene is the only or key target of a chromosomal alteration, or that it is involved in cancer pathogenesis.

ABCA4 [27], *TAX1BP1* [28], *GRHL2* [29], *PLEKHA5* [30], and *MTMR3* [31]. We detected the rate of recurrent aberrated chromosomal region using CNAG. The rates of recurrent aberrant chromosomal regions were 41.2% (*PTCH2* and *MTMR3*), 52.9% (*GRHL2*, *CCND1*, *PPFIBP1*, *PLEKHA5*, and *PRKCM*), 58.8% (*ABCA4* and *TAX1BP1*), and 64.7% (*MAPK7*).

Quantitative real-time PCR showed that the copy number of most of the regions estimated by HMM was greater than five (Table 2). The shortest region of high-grade gain was 129 kb, spanning positions 19.432–19.561 Mb on 17p11.2. The total number of genes in the regions of high-grade gain ranged from 1 (12p12.3, 19.118–19.295 Mb) to 15 (11q13.2-3, 68.089–69.728 Mb, 14q12, 28.090–30.519 Mb). The regions 11q13.2-3 and 12p12.3 showed high-grade gain in three cell lines, and the remaining regions showed high-grade gain in two cell lines. Of the 10 regions, 11q13.2-3, which includes *CCND1*, was previously reported to be amplified or gained at the genome level [22], but the remaining 9 regions were newly detected as candidates for high-grade gain at the nucleotide level.

3.4. Association between gain of genes and clinico-pathological features

There is much evidence that gain and amplification of oncogenes can lead to cancer. To analyze the involvement of oncogenes in carcinogenesis, we focused on the analysis of copy number gain in this study. To quantify the copy number of 10 genes, quantitative real-time PCR was performed on 70 HCC clinical samples. Baseline characteristics of 70 patients were summarized in Table 3.

Table 4 shows the results of amplification frequency compared to non-cancerous tissue, Edmondson grade I (Ed I) HCC, and Ed II or III HCC. Overall, the copy number was almost normal in non-cancerous tissue and frequently amplified in Ed II or III HCC compared to Ed I HCC.

We further examined the associations with other clinico-pathological parameters. A gain of 10 genes was assessed among subgroups divided by age (over vs. under 65 years), sex (male vs. female), etiology (HCV vs. non-HCV), liver function (Child-Pugh class A vs. class B or C), size of tumors (over vs. under 3 cm),

Table 3
Baseline characteristics of 70 patients

Variables	N (%)
Mean age (yrs)	64.5 ± 9.3
Sex	
Male	53 (75.7%)
Female	17 (24.3%)
Etiology	
HBs Ag positive	14 (20.0%)
HCV Ab positive	47 (67.1%)
Both negative	9 (12.9%)
Child-Pugh class	
A	38 (61.3%)
B or C	24 (38.7%)
Size of tumors (cm)	2.6 ± 2.1
Number of tumors	
Solitary	45 (64.3%)
Multiple	25 (35.7%)
Edmondson grade	
I	22 (31.4%)
II or III	48 (68.6%)
Background	
Non-cirrhosis	21 (30.0%)
Liver cirrhosis	49 (70.0%)

number of tumors (solitary vs. multiple), pathologic grade of tumors (Ed I vs. Ed II or Ed III), and background (non-cirrhosis vs. cirrhosis).

As shown in Table 5, the copy number gain of *TAX1BP1* in 7p15.2-1 is associated with positivity for HCV antibody ($p = 0.0279$) and larger tumor size ($p = 0.0216$) by univariate analysis. That of *GRHL2* in 8q22.3 is associated with larger tumor size ($p = 0.0216$) and poor differentiation ($p = 0.0062$), and *CCND1* in 11q13.2-3 is also associated with larger tumor size ($p = 0.0216$) and poor differentiation ($p = 0.0212$). Multivariate analysis revealed that the copy number gain of *TAX1BP1* is associated with HCV antibody positivity

Table 4
Frequency of copy number gain (≥3) in hepatocellular carcinoma samples

	Non-cancerous tissue ($N = 15$)	Edmondson grade I ($N = 22$)	Edmondson grade II, III ($N = 48$)
PTCH2	1 (6.7%)	3 (13.6%)	8 (16.7%)
ABCA4	0 (0.0%)	6 (27.3%)	10 (20.8%)
TAX1BP1	1 (6.7%)	5 (22.7%)	17 (35.4%)
GRHL2	1 (6.7%)	2 (9.1%)	20 (41.7%)
CCND1	0 (0.0%)	2 (9.1%)	18 (37.5%)
PPFIBP1	0 (0.0%)	2 (9.1%)	10 (20.8%)
PLEKHA5	1 (6.7%)	4 (18.2%)	12 (25.0%)
PRKCM	0 (0.0%)	2 (9.1%)	14 (29.2%)
MAPK7	0 (0.0%)	5 (22.7%)	12 (25.0%)
MTMR3	1 (6.7%)	2 (9.1%)	9 (18.8%)

Table 5
Factors associated with gain of *TAX1BP1*, *GRHL2*, *CCND1* and clinico-pathological parameters (univariate analysis)

Variable	Copy number < 3 ($N = 48$)	Copy number ≥ 3 ($N = 22$)	p Value*
(A) TAX1BP1			
Age (yrs)			
≥65	24 (50%)	13 (59%)	
<65	24 (50%)	9 (41%)	0.6075
Sex			
Male	34 (71%)	19 (86%)	
Female	14 (29%)	3 (14%)	0.2324
Etiology			
HCV Ab positive	28 (58%)	19 (86%)	
HCV Ab not-positive	20 (42%)	3 (14%)	0.0279
Liver function			
Child-Pugh A	33 (69%)	13 (59%)	
Child-Pugh B, C	15 (31%)	9 (41%)	0.5947
Size of tumors (cm)			
≥3	18 (38%)	15 (68%)	
<3	30 (62%)	7 (32%)	0.0216
No. of tumors			
Solitary	31 (65%)	14 (64%)	
Multiple	17 (35%)	8 (36%)	1.0000
Histology			
Edmondson I	17 (35%)	5 (23%)	
Edmondson II, III	31 (65%)	17(77%)	0.4072
Background			
Chronic hepatitis	11 (23%)	10 (45%)	
Liver cirrhosis	37 (77%)	12 (55%)	0.0904
(B) GRHL2			
Age (yrs)			
≥65	25 (52%)	12 (55%)	
<65	23 (48%)	10 (45%)	1.0000
Sex			
Male	35 (73%)	18 (82%)	
Female	13 (27%)	4 (18%)	0.5531
Etiology			
HCV Ab positive	32 (67%)	15 (68%)	
HCV Ab negative	16 (33%)	7 (32%)	1.0000
Liver function			
Child-Pugh A	31 (65%)	15 (68%)	
Child-Pugh B, C	17 (35%)	7 (32%)	1.0000
Size of tumors (cm)			
≥3	18 (38%)	15 (68%)	
<3	30 (62%)	7 (32%)	0.0216
No. of tumors			
Solitary	34 (71%)	11 (50%)	
Multiple	14 (29%)	11 (50%)	0.1117
Histology			
Edmondson I	20 (42%)	2 (9%)	
Edmondson II, III	28 (58%)	20 (91%)	0.0062
Background			
Chronic hepatitis	15 (31%)	6 (27%)	
Liver cirrhosis	33 (69%)	16 (73%)	0.7866

Table 5 (*continued*)

Variable	Copy number < 3 (N = 48)	Copy number ≥ 3 (N = 22)	p Value*
(C) CCND1			
Age (yrs)			
≥65	31 (62%)	16 (80%)	
<65	17 (38%)	6 (20%)	0.1715
Sex			
Male	35 (70%)	18 (90%)	
Female	13 (30%)	4 (10%)	0.5531
Etiology			
HCV Ab positive	32 (64%)	15 (75%)	
HCV Ab negative	16 (36%)	7 (25%)	1.0000
Liver function			
Child-Pugh A	31 (66%)	15 (65%)	
Child-Pugh B, C	17 (34%)	7 (35%)	1.0000
Size of tumors (cm)			
≥3	18 (36%)	15 (75%)	
<3	30 (64%)	7 (25%)	0.0216
No. of tumors			
Solitary	34 (72%)	11 (50%)	
Multiple	14 (28%)	11 (50%)	0.1117
Histology			
Edmondson I	18 (40%)	4 (10%)	
Edmondson II, III	30 (60%)	18 (90%)	0.0212
Background			
Chronic hepatitis	10 (24%)	11 (50%)	
Liver cirrhosis	38 (76%)	11 (50%)	0.0938

* Fisher's exact test.

($p = 0.0146$) and larger tumor size ($p = 0.0099$), and that of *CCND1* is associated with larger tumor size ($p = 0.0113$) (Table 6). The size of tumors and histology were not significant independent factors in the gain of *GRHL2*.

Table 6
Factors associated with gain of *TAX1BP1*, *GRHL2*, *CCND1* and clinico-pathological parameters (multivariate analysis)

Variable	Odds ratio (95% CI)	p Value*
(A) TAX1BP1		
HCV Ab positive	5.908 (1.420–24.588)	0.0146
Size of tumors (≥3 cm)	4.554 (1.439–14.409)	0.0099
(B) GRHL2		
Size of tumors (≥3 cm)	2.148 (0.671–6.880)	0.1979
Edmondson II, III	5.004 (0.957–26.158)	0.0564
(C) CCND1		
Size of tumors (≥3 cm)	5.543 (1.473–20.854)	0.0113
Edmondson II, III	2.770 (0.499–15.391)	0.2441

CI, confidence interval.
* Logistic regression.

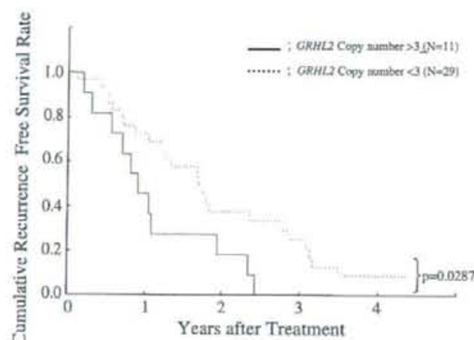

Fig. 3. Cumulative probabilities of recurrence-free survival after local ablation therapy for HCC estimated by Kaplan–Meier analysis: *GRHL2* gain (copy number ≥ 3) group (line) and non-gain (copy number < 3) group (dashed line) are shown.

3.5. Association between gain of genes and recurrence-free survival

We next investigated the association between gain of genes and recurrence-free survival. Samples from 40 patients treated with ablation therapy were analyzed with the Kaplan–Meier model for subgroups, including gain (copy number ≥ 3) of 10 genes, because they had precise recurrence data of HCCs.

The Kaplan–Meier method revealed that the gain of *GRHL2* in 8q22.3 was significantly associated with recurrence-free survival, and other parameters were not (Fig. 3). Furthermore, the effects of clinical features considered to be associated with recurrence-free survival were assessed by multivariate Cox proportional hazard regression; these included histology of tumor, tumor size, and number of recurrence to control clinical parameters. A gain of *GRHL2* had a statistically significant hazard ratio 2.621 (95% CI: 1.118–6.145; $p = 0.0267$; Table 7), and gain of *GRHL2* was the most significant factor for HCC recurrence among them. These results suggest that a gain of *GRHL2* might be an independent predictive marker for HCC recurrence.

Table 7
Multivariate analysis with respect to recurrence-free survival

Variable	Hazard ratio (95% CI)	p Value*
Edmondson II, III	1.261 (0.591–2.693)	0.5482
Tumor size (≥3 cm)	1.038 (0.466–2.312)	0.9269
Non-Naïve case	1.916 (0.868–4.228)	0.1072
Gain of GRHL2	2.621 (1.118–6.145)	0.0267

* Cox proportional hazard regression model.

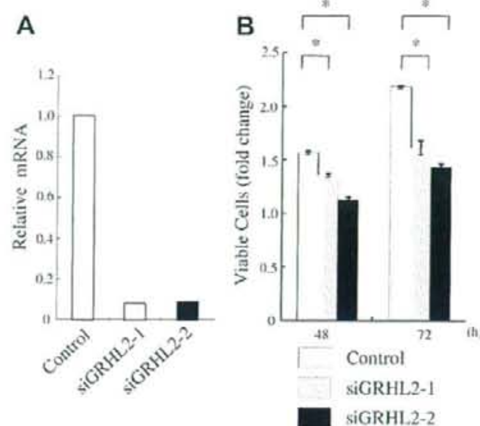

Fig. 4. Suppression of hepatoma cell growth induced by *GRHL2* knockdown. *GRHL2* expression 48 h after transfection with the siGRHL2-1 and siGRHL2-2 vector analyzed by quantitative reverse transcription-PCR. (B) Proliferation of HuH-6 cells by siGRHL2-1 and siGRHL2-2 transfection, 48 and 72 h after reseeding. Mean fold-changes relative to the control (0 h) from three independent experiments are shown. Bars, standard deviation. $^{*}p < 0.05$. Representative data from one of three independent experiments.

3.6. Decreased GRHL2 expression by RNA interference inhibits the growth of hepatoma cells

To further elucidate the association between the *GRHL2* gene and recurrence-free survival, *GRHL2* function in cell proliferation was investigated using RNA interference. HuH-6, in which *GRHL2* was amplified and overexpressed, was used for analysis. The reduction of endogenous *GRHL2* expression in HuH-6 cells was confirmed by quantitative reverse transcription-PCR (Fig. 4A). The proliferation assay revealed that *GRHL2* knockdown markedly repressed HuH-6 cell growth (86% and 72% after 48 h, $p < 0.05$, and 74% and 66% after 72 h, $p < 0.05$; Fig. 4B) compared to the control HuH-6 cells. These results suggest that *GRHL2* is involved in cell proliferation.

4. Discussion

In this study, we surveyed 17 hepatoma cell lines using Affymetrix GeneChip Human Mapping 50 K arrays and a data analysis tool, CNAG, for determining chromosomal imbalance. Furthermore, we assessed the association between a gain of 10 genes and clinico-pathological features, and showed the possibility that a gain of *GRHL2* is a novel genetic marker to predict HCC recurrence.

The genomes of hepatoma cell lines were broadly amplified due to polyploidity. However, the tendency for a gain and loss of chromosomes was essentially consistent with previous reports [32,33] and a database on the Web (http://www.cghtmd.jp/CGHDatabase/index.html). The high frequency LOH regions detected by GeneChip included known targets for LOH, *DLC1* [34], *Rb1* [35],*CDKN2A* [36], and *TP53* [37]. A gain region is defined as an increased allele compared to the baseline of one chromosome. Several studies, however, have reported that patients with polyploidy have a poorer prognosis compared to those with euploidy [38–40]; thus, not only an increased allele compared to the baseline of one chromosome, but also an increased allele itself can impact cancer analysis. To clarify this point, in this study, we analyzed the absolute copy number of entire chromosomes (Fig. 1) and selected the region above copy number 3 for further analysis. We measured DNA content by FACS and exact copy number using these data, which are very useful tools for analyzing the function of novel putative oncogenes and TSGs.

To identify regions that might contain putative oncogenes, we chose 10 genes from recurrent high-grade gains of hepatoma cell lines. We used criteria for choosing the genes to efficiently analyze the oncogenes, because the gain region span was too broad to analyze in the cell lines. Among them, we selected a known HCC amplification target, *CCND1*, as a positive control. *CCND1* is amplified and overexpressed in 10 to 15% of HCC samples and is considered to result in the rapid growth of a subset of HCC [22,23].

We examined the association between a gain of 10 genes and clinico-pathological parameters. An association between *CCND1* and the size of tumors is reasonable [23], however, an association between *TAX1BP1* and the size of tumors and HCV infection is unclear. *TAX1BP1* codes for TAX1-binding protein 1, which is reported to be a binding partner of TAX1 and is involved in anti-apoptosis [28], therefore HCC with gain of *TAX1BP1* might be advantageous for growth.

In our study, recurrence-free survival was associated with *GRHL2*, which is a grainyhead-like 2 gene that codes for CP2-like 3 (TFCP2L3), and considered to be a putative transcription factor. A genome-wide scan of a large American family with an autosomal dominant form of progressive, non-syndromic, sensorineural hearing loss revealed that a heterozygous mutation of *TFCP2L3* results in hearing loss, although its function remains unknown [29]. In this study, decreased *GRHL2* expression by RNA interference inhibited the growth of hepatoma cells, suggesting its association with cell proliferation. The amplification of 8q in HCC was described in many reports with high frequency [11–13,41–44], however, this is the first report that revealed the associ-

ation between gain of *GRHL2* in 8q22.3 and early HCC recurrence. To validate these results, we need to investigate other patient cohorts.

Discriminating intrahepatic metastasis, local recurrence, and multicentric hepatocarcinogenesis in strict terms is difficult. However, at our institute, we always confirm that the entire tumors are ablated with a safety margin by RFA treatment; therefore, local recurrence is rare (2.6% cumulative incidence in our study [3]), and the samples with local recurrence were not included in this study. Furthermore, early recurrence, especially within 1–3 years after treatment, is considered to arise mainly due to intrahepatic metastasis [45–47]. The majority of the patients with *GRHL2* gain tumors resulted in early recurrence within 2 years. Therefore, we postulate that the majority of the recurrent tumors had the same clonality as the initial tumors with *GRHL2* gain, which resulted in intrahepatic metastasis and early recurrence.

Our study has several limitations. First, we did not use fresh frozen tissue for SNP array analysis. We adopted the strategy to investigate allelic imbalance by SNP array using hepatoma cell lines and to validate the results using clinical samples. One of the aims of this study is to find novel genetic marker associated with clinical features, therefore we used FFPE samples with detailed clinical data, from which we could only obtain small amount and lower quality of DNA for array analysis. Second, the 10 genes were arbitrarily selected and analyzed. To enhance the possibility to find the regions that contain oncogenes, we selected 10 narrow amplified regions for analysis and represented these regions with 10 genes. Although the possibility exists that we missed true oncogenes in other amplified regions, our results show that the gain of frequency is relatively high in all the regions in the clinical HCC samples (16–41% in Ed II HCC). Third, we got centered on the analysis of gain regions in this study, and did not investigate deleted and LOH regions in clinical specimens. The association between clinico-pathological features and the deleted region is now under investigation. Fourth, we used 10 genes as molecular markers and did not examine the expression and function of these genes. We need further investigation to pinpoint the genes that are the target of amplification, deletion and LOH.

Though these limitations exist, one of the merits of copy number quantification using real-time PCR in this study is that it can be performed with a small amount of DNA. This method is widely applicable to clinical samples, even FFPE specimens obtained by liver biopsy, for prediction of HCC recurrence.

In summary, we identified chromosomal alterations associated with clinico-pathological parameters using the SNP array data. The patients with HCC of *GRHL2* gain should be followed up closely and, if recurrence occurred, we need to choose appropriate therapeutic options for these patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2008.06.019.

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Risk Assessment of Hepatocellular Carcinoma in Chronic Hepatitis C Patients by Transient Elastography

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Objective: The degree of liver fibrosis is the strongest indicator of risk for hepatocellular carcinoma (HCC) development. Recently developed transient elastography (Fibroscan, Echosens, France) noninvasively measures liver stiffness, and the correlation between the stiffness and liver fibrosis stage has been validated. In this cross-sectional study, we investigated the relationship between liver stiffness and HCC presence.

Methods: Liver stiffness was measured in chronic hepatitis C patients (85 with HCC and 180 without) by transient elastography. Multivariate logistic regression was applied to assess the association with HCC presence. We computed the receiver operating characteristics (ROC) curves concerning the prediction of HCC presence and compared the areas under ROC curve (AUROC). We also calculated stratum-specific likelihood ratios (SSLR).

Results: Multivariate analysis showed that HCC presence was significantly associated with liver stiffness ($P < 0.0001$) along with age, male, and alpha-fetoprotein concentration. AUROC was 0.805, 0.741, 0.714, 0.673, 0.670, and 0.654 for liver stiffness, alpha-fetoprotein, albumin, prothrombin activity, AST-platelet ratio index, and platelet count, respectively. Other parameters showed smaller AUROC. SSLR for HCC presence by liver stiffness was 0.22 (95% confidence interval: 0.11-0.42) in < 10 kPa, 0.73 (0.39 to 1.39) in 10.1 to 15 kPa, 1.30 (0.80 to 2.12) in 15.1 to 25 kPa, and 5.0 (2.96 to 8.47) in > 25 kPa.

Conclusions: Liver stiffness measured by transient elastography is useful in demarcating chronic hepatitis C patients at a high risk for HCC, who require frequent check-up by imaging examinations.

Key Words: liver fibrosis, receiver operating characteristics (ROC), stratum-specific likelihood ratio (SSLR)

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Hepatocellular carcinoma (HCC) is a common malignancy worldwide.[1] The incidence of HCC is particularly high in geographical areas where there is the prevalence of hepatitis B or hepatitis C virus infection. HBV infection is extremely prevalent in some Asian and African countries whereas HCV infection is prevalent in Japan and southern European countries. In these countries chronic viral hepatitis is the major cause of HCC, the incidence of HCV-related HCC is recently increasing also in the Unites States and some other countries.[2-4]

In the natural history of hepatitis C, HCC emerges after long duration of infection for more than 20 years, ordinarily through cirrhosis.[5-8] Liver fibrosis, which is a consequence of inflammation and regeneration, is thought to be a surrogate marker of accumulated damage to DNA. Indeed, the degree of fibrosis is reported to be the strongest indicator of risk for HCC development.[9] Until recently, the degree of liver fibrosis could be assessed only with liver biopsy. However, extensive data on liver biopsy indicated that severe complications occur in 3%, including death in 0.03%.[10]

A recently introduced technique, transient elastography, noninvasively measures hepatic elasticity, or stiffness.[11] An elastic shear wave is generated by a special vibrator and its propagation velocity in the liver is measured with pulse-echo ultrasound. Because the propagation velocity of shear waves is directly proportional to the square root of tissue stiffness (elastic modulus), we can measure the stiffness of liver. Liver fibrosis is considered to be the largest contributor to the liver stiffness thus measured. Recent reports confirmed the correlation between the degree of liver fibrosis and the liver stiffness in chronic hepatitis C patients.[12-15]

Thus, we can assume that the liver stiffness is closely related to the risk of HCC development, although this is yet to be confirmed in future prospective studies. In this study, we sought to validate the relationship between liver stiffness and the presence of HCC in a cross-sectional study determining the liver stiffness in 85 HCV-related patients and 180 chronic hepatitis C patients without HCC.

PATIENTS AND METHODS

Patients

From March 2005 to October 2005, a total of 92 HCV-related naive HCC patients were admitted to the

authors' hospital. The liver stiffness was measured by transient elastography in 85 patients as written below, excluding 4 patients with massive HCC that occupied substantial portion of the right lobe, and other 3 patients with ascites because fluid inhibits the measurement of stiffness by terminating the propagation of elastic shear wave. During the corresponding period, liver stiffness was measured in consecutive 195 outpatients with chronic hepatitis C without HCC. Because all HCC patients were older than 52 years, 180 patients without HCC who were older than 52 years were selected as controls. The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration and was approved by the institutional review board. Patients were enrolled with written informed consent.

Diagnosis of HCC

HCC was diagnosed by dynamic computed tomography, where intrahepatic nodules with hyperattenuation in the arterial phase with washout in the late phase were considered as definite HCC.[16] Histopathologic diagnosis, using ultrasound-guided biopsy samples, was also performed when required. HCC was ruled out by ultrasound in each control patient. No HCC was detected in the subsequent 6-month follow-up period among the patients without HCC.

Transient Elastography

Fibroscan (EchoSens, Paris, France) is a medical device specifically designed for hepatic transient elastography.[11–14] An ultrasonic transducer is mounted on the axis of a vibrator that induces an elastic shear wave into the liver and its propagation velocity was measured by pulse-echo ultrasound acquisitions. The procedure is to be performed on the right lobe of the liver through an intercostal space, where intrahepatic transit is at least 6 cm thick free of large vascular structures. Ten valid measurements were performed on each patient and the median value was recorded.

Transient elastography cannot be applied to patients with ascites because elastic waves do not propagate through liquids. In addition, liver elastography may be unsuccessful in patients with morbid obesity, although there were no such patients in the current study period. Foucher et al[17] assessed the prevalence and factors associated with the failure of liver stiffness measurement in 2114 patients by multivariate analysis and body mass index was the only factor associated with failure.

Biomarkers

We determined the following parameters on the day of liver stiffness measurement: serum albumin and total bilirubin concentrations, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, prothrombin activity, serum alpha-fetoprotein (AFP) concentration, and platelet cell count. We also assessed AST-platelet ratio index (APRI) as APRI = AST (× upper limit of normal) × 100/platelet count (10^9/L).[18]

ROC Analysis

In receiver operating characteristic (ROC) curves, the true-positive rate (test sensitivity) is plotted as a function of the false-positive rate (1-test specificity) while moving the cut-off through its possible range. We calculated ROC curves assessing the risk of HCC presence via liver stiffness measured by transient elastography and the biomarkers. The area under the ROC curve (AUROC) is the most commonly used method of summarizing the overall accuracy, where an area of 1 represents a perfect test and an area of 0.5 means a noninformative test. We computed and compared AUROC for each parameter.

Stratum-specific Likelihood Ratio

ROC curves are usually used to select the optimal cut-off that plots the point nearest to the upper left corner. However, our intention was not to diagnose HCC by surrogate tests but to estimate the probability of HCC presence in each patient, and in clinical practice diagnostic imaging should be performed accordingly. In this respect, continuous evaluation of the risk is much preferable to a determination by a single fixed cut-off.

According to Bayes' theorem, posttest odds [= probability/(1 − probability)] is the product of pretest odds and the likelihood ratio. Stratum-specific likelihood ratio (SSLR) is defined as a ratio of 2 probabilities, the probability of a test result within a given range, or stratum, when the disease is present, divided by the probability of the same test result when the disease is absent. By using SSLR, we can estimate the posttest probability for any given test result. SSLR was calculated as the proportion of diseased subjects with a test result in a given range divided by the proportion of nondiseased subjects with a test result in the same given range.[19–22] The 95% confidence interval of each SSLR was calculated according to the method proposed by Peirce et al.[22]

Statistical Procedures

The association between the presence of HCC and other factors including liver stiffness was assessed by univariate and multivariate analyses. In univariate analysis categorical variables were compared by χ^2 tests, whereas continuous variables were compared with unpaired student t test. The following variables were analyzed after logarithmic transformation for normality of distribution: liver stiffness, total bilirubin, AST, ALT, and APRI. Unconditional logistic regression was used in multivariate analysis. A 2-sided P value of < 0.05 was considered statistically significant. Statistical analysis was performed by SPSS software version 9.0 (SPSS, Inc, Chicago, IL) and S-plus 2000 (MathSoft, Inc, Seattle, WA).

RESULTS

Characteristics of the Patients

The demographics of HCV-related HCC patients ($n = 85$) and chronic hepatitis C patients without HCC

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(n = 180) were summarized in Table 1. We limited the study subjects to naive HCC cases to avoid possible influence of previous treatments. Reflecting the current epidemiologic status of HCC in Japan, HCC patients were aged near 70 and male dominant. The median size of HCC nodules were 2.5 cm in diameter (ranged from 1.8 to 5.6 cm). HCC was uninodular in 41 patients, 2 nodules in 25 patients, 3 nodules in 7 patients, and more than 3 in 10 patients. AFP was > 20 ng/mL in 41 patients (48.2%) and > 100 ng/mL in 20 (23.5%). In chronic hepatitis C patients without HCC, AFP was > 20 ng/mL in 25 (13.9%) and > 100 ng/mL in 2 (1.1%). Platelet count, albumin concentration, and prothrombin activity were lower among the patients with HCC than among those without. The difference in liver stiffness was discussed below.

Logistic Regression Analysis

Association with the presence of HCC was assessed by univariate analysis for each factor as shown in Table 1. Subsequent multivariate analysis showed that age [odds ratio (OR): 1.05 by 1 y, 95% confidence interval (CI): 1.00-1.10, $P = 0.0028$], male (OR: 3.38 vs. female, 95% CI: 1.70-6.71, $P = 0.0023$), AFP > 100 ng/mL (OR: 2.24, 95% CI: 1.06-4.72, $P = 0.034$), and $\log_{10}$ (liver stiffness) (OR: 25.0, 95% CI: 6.37-98.1, $P < 0.001$) were independent predictors of HCC presence.

ROC Curves

The scatter diagram of the liver stiffness measured by transient elastography showed that patients with HCC had larger values of stiffness (Fig. 1). By changing the cut-off value, we plotted an ROC curve for liver stiffness regarding the presence of HCC. ROC curves were also plotted for other laboratory parameters. AUROC was the largest for liver stiffness (0.805), followed by AFP (0.741), albumin (0.714), prothrombin activity (0.673), APRI (0.670), and platelet count (0.654) (Fig. 2). AUROCs for bilirubin, ALT, and AST were smaller than 0.6 and their ROC curves were omitted from the graph.

SSLR

Although there are no definite rules relating to the number of strata in SSLR, using too many strata may result in inaccurate estimation of likelihood ratios. We arbitrarily ranked the values of liver stiffness into 4 strata. SSLR for the presence of HCC was 0.22 (95% CI: 0.11-0.42) when liver stiffness was in the range < 10 kPa, 0.73 (0.39 to 1.39) in 10.1 to 15 kPa, 1.30 (0.80 to 2.12) in 15.1 to 25 kPa, and 5.0 (2.96 to 8.47) in > 25 kPa (Table 2).

TABLE 1. Characteristics of Patients*

Variables	Patients Without HCC (N = 180)	Patients With HCC (N = 85)	P
Age, y	70.8 (64.7-75.3)	67.5 (61.3-72.4)	0.0028
Male/female, n/n	79/101	54/31	0.0027
Platelet count (10^9/L)	145 (105-186)	103 (74-156)	0.00017
Bilirubin (mg/dL)	0.8 (0.6-1.0)	0.8 (0.6-1.2)	< 0.0001
Albumin (g/dL)	4.0 (3.7-4.2)	3.6 (3.2-3.9)	< 0.0001
AST (IU/L)	43 (31-64)	53 (41-74)	0.0044
ALT (IU/L)	45 (30-70)	48 (31-62)	0.22
PT (%)	82.9 (73.9-97.1)	74.0 (67.3-83.3)	< 0.0001
AFP (ng/mL)	5 (3-11)	18 (6-93)	< 0.0001
APRI	0.84 (0.50-1.48)	1.49 (0.88-2.51)	< 0.0001
Stiffness (kPa)	9.85 (5.8-16.8)	22.3 (14.4-36.3)	< 0.0001

*Expressed as median (25th to 75th percentiles).

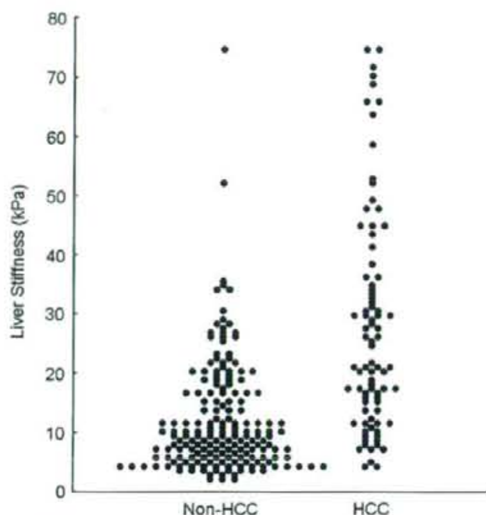

FIGURE 1. Frequency distribution of liver stiffness values in kPa. Liver stiffness is greater in the patients with HCC than in those without.

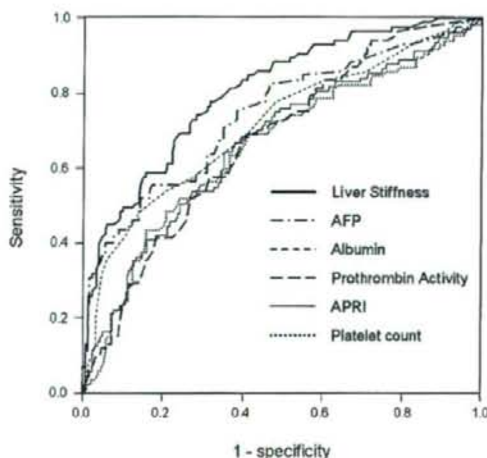

FIGURE 2. ROC curves for liver stiffness, AFP, albumin, PT, APRI, and platelet. The areas under the ROC curves are 0.805, 0.741, 0.714, 0.673, 0.670, and 0.654, respectively.

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TABLE 2. Stratum-specific Likelihood Ratio Analysis of Liver Stiffness

	No. Patients		
Strata (kPa)	Non-HCC (N = 180)	HCC (N = 85)	SSLR (95% CI)
< 10	94	10	0.22 (0.11-0.42)
10.1-15	34	12	0.73 (0.39-1.39)
15.1-25	34	21	1.30 (0.80-2.12)
> 25	18	42	5.00 (2.96-8.47)

This indicates that posttest odds for HCC presence increase 5-fold when liver stiffness is larger than 25 kPa whereas the odds decrease to one-fifth when stiffness is smaller than 10 kPa.

DISCUSSION

Risk factors for HCC development among chronic liver disease patients have been examined in several cohort studies.[7,9,23–26] In a cohort study among 2800 chronic hepatitis C patients, Yoshida et al reported the degree of liver fibrosis, age, and male sex to be risk factors for HCC development, among which liver fibrosis was by far the strongest. Velazquez et al[23] analyzed 463 patients with cirrhosis of various etiologies and observed that age, prothrombin activity, platelet cell count, and anti-HCV seropositivity were independently associated with the development of HCC. Other factors reportedly associated with the risk of HCC development include high serum ALT, high serum AFP,[26] and heavy alcohol consumption. Although other parameters are readily available, the assessment of liver fibrosis used to require liver biopsy. The close association has been recently established between the degree of liver fibrosis and liver stiffness measured by transient elastography.

With transient elastography, liver stiffness is expressed as a continuous variable. This may provide an advantage over histologic distinction between cirrhosis and noncirrhosis because noncirrhotic liver, at fibrosis stage F3 in particular, still possesses a definite risk of HCC development.[9] Moreover, transient elastography may be able to differentiate risk of HCC among cirrhotic livers. Although the cut-off value for cirrhosis is usually set at around 15 kPa,[12–14] our current study has shown that liver stiffness > 25 kPa, which indicates "stiffer cirrhosis," has the strongest association with the presence of HCC. Liver stiffness may be proportional to the risk of HCC even within the range of cirrhosis, which should be confirmed in prospective studies.

In multivariate analysis, 4 parameters, namely, liver stiffness, age, male, and AFP, were associated with the presence of HCC. Other parameters such as albumin, platelet count, and APRI were not retained as significant. This probably indicates that these parameters were so strongly associated with liver fibrosis, and thus with liver stiffness, that they did not provide information on HCC presence supplementing liver stiffness. On the other hand, AFP seems to be a risk factor independent of liver stiffness.

In AUROC analysis, liver stiffness surpassed AFP in the accuracy of HCC prediction. This may seem to be paradoxical because liver stiffness is only an indirect measurement of risk of HCC whereas AFP is a biomarker specific to HCC. AFP has served as a diagnostic test for HCC since the 1970s. However, AFP level may be normal in 35% to 45% of HCC patients, and the sensitivity may be much smaller in patients with small HCC,[27,28] such as the majority of cases in the current study. On the other hand, patients with cirrhosis or chronic hepatitis without HCC may show an elevated AFP level probably due to inflammation and hepatocytic regeneration. However, this may reflect a true risk of future HCC development and, indeed, Colombo et al[26] showed that cumulative hazard of HCC development during follow-up was higher among patients with persistently elevated AFP levels than among those with fluctuating or consistently normal levels.

In clinical practice, liver stiffness will not be used as a diagnostic test for HCC but as an indicator of the risk of HCC. In this respect, SSLRs have several advantages over prediction by a fixed cut-off. For continuous scores such as liver stiffness, SSLRs retain as much information as possible by deriving multiple level indices. Although previous study indicated 12 to 15 kPa as the cut-off for cirrhosis, the current study demonstrated that likelihood ratio for HCC differs according to liver stiffness even within this range. In the current study, posttest odds for HCC presence increase 5-fold when stiffness is larger than 25 kPa whereas decrease to one-fifth when it is smaller than 10 kPa, and the posttest probability can be obtained with a simple nomogram.[29] The prevalence of target disease at each clinic may be used as the pretest probability. Likelihood ratio, a ratio of 2 probabilities, is not influenced by the proportion of patients with disease in a given population. Thus, the results obtained in this study are applicable to any population provided that background characteristics are similar.

Although risk ratios cannot be obtained in cross-sectional studies, likelihood ratios are known to approximate risk ratios. The likelihood ratio between > 25 and < 10 kPa was 22.7 in the current study, which was similar to a prospectively observed risk ratio of 24.0 between fibrosis stages 4 and stage 1.[9] Another limitation of the current study is that we cannot estimate future changes in the risk of HCC. Although progression of fibrosis is generally gradual in chronic hepatitis C, the progression rate varies among individual patients.[6,8] The factors not found associated with the presence of HCC in this study, such as AST and ALT, may still be a risk factor of HCC by affecting fibrosis progression rates.

Transient elastography has the distinct advantage of being noninvasive. In clinical practice, transient elastography will be applied primarily on chronic hepatitis C patients possibly, but not definitely, with advanced liver fibrosis. In place of liver biopsy, noninvasive measurement of liver stiffness can distinguish patients with "stiff"

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liver who are at a high risk of HCC development and should be surveyed regularly with imaging studies. Another interesting application will be the long-term monitoring of stiffness progression in patients with "soft" liver at present.

In conclusion, liver stiffness measured by transient elastography is useful in demarcating chronic hepatitis C patients at a high risk for HCC development, who require frequent check-up by imaging examinations. Even if HCC is negative, careful monitoring will be necessary for these patients.

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Safety and efficacy of lamivudine after radiofrequency ablation in patients with hepatitis B virus-related hepatocellular carcinoma

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Abstract

Background Antiviral treatments for hepatitis B virus (HBV) are not established in patients with HBV-related hepatocellular carcinoma (HCC).

Aim To investigate the safety and efficacy of lamivudine (LAM) in patients with HBV-related HCC who were treated with radiofrequency ablation (RFA).

Methods RFA-treated patients with HBV-related HCC were retrospectively divided into those who received LAM (LAM group) and those who did not (nontreatment group). The first-year changes in serum alanine aminotransferase (ALT), total bilirubin (TBIL), and albumin (ALB) levels were compared in corresponding subsets based on Child-Pugh classification (Mann–Whitney U test) and between one-to-one matched pairs (Wilcoxon signed rank test), who were selected on the basis of their propensity scores for receiving LAM. Overall and recurrence-free survival was also compared.

Results Complete ablation of HCC was achieved in 104 patients with HBV-related HCC between January 2000 and December 2005. LAM was administered to 33 patients after RFA. Serum HBV-DNA became negative by TMA method in 24 (73%) patients. Four patients showed redetection of HBV-DNA with ALT elevation. Subset analysis based on initial Child-Pugh class and paired analysis with matching revealed significant decreases in ALT and bilirubin levels and increases in ALB levels in the first year in the LAM group ($\Delta ALT = -17$, $\Delta ALB = +0.3$, and $\Delta TBIL = -0.2$) compared with controls ($\Delta ALT = +5$, $\Delta ALB = \pm 0.0$, and $\Delta TBIL = +0.3$). Overall survival and recurrence-free survival did not differ between the two groups. No specific adverse effect was observed in the LAM group.

Conclusion LAM after RFA for HBV-related HCC was safe and improved liver function. Further studies are needed to evaluate its effect on survival.

Keywords Hepatitis B · Hepatocellular carcinoma · Lamivudine · Radiofrequency ablation

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Introduction

Hepatocellular carcinoma (HCC) is one of the major consequences of chronic hepatitis B virus (HBV) infection [1–3]. The prevalence of HBV infection widely differs geographically, and is very high in many countries in East Asia and sub-Saharan Africa. Those countries also show a high incidence of HBV-related HCC. Surgical resection can be performed for HCC at the early stages, but is frequently contraindicated by background cirrhosis common in HCC patients. Although liver transplantation is a potential treatment for HCC with liver dysfunction, its feasibility is limited by the scarcity of donor organs. Radiofrequency ablation (RFA) is an alternative treatment for HCC, applicable to patients with moderately impaired liver function [4–10]. The prognosis after RFA is reported to be comparable to that after surgical resection.

Patients with HBV-related HCC treated with RFA are to be confronted with two serious problems: recurrence of HCC and exacerbation of liver dysfunction. Although recurrent HCC may be treated with repeated RFA or other therapeutic modalities, additional treatments may be prohibited by further deterioration in liver function. Moreover,