Alteration of Gene Expression in Human Hepatocellular Carcinoma with Integrated Hepatitis B Virus DNA

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Abstract

Purpose: Integration of hepatitis B virus (HBV) DNA into the human genome is one of the most important steps in HBV-related carcinogenesis. This study attempted to find the link between HBV DNA, the adjoining cellular sequence, and altered gene expression in hepatocellular carcinoma (HCC) with integrated HBV DNA.

Experimental Design: We examined 15 cases of HCC infected with HBV by cassette ligation–mediated PCR. The human DNA adjacent to the integrated HBV DNA was sequenced. Protein coding sequences were searched for in the human sequence. In five cases with HBV DNA integration, from which good quality RNA was extracted, gene expression was examined by cDNA microarray analysis.

Results: The human DNA sequence successive to integrated HBV DNA was determined in the 15 HCCs. Eight protein-coding regions were involved: ras-responsive element binding protein 1, calmodulin 1, mixed lineage leukemia 2 (MLL2), FLJ333655, LOC220272, LOC255345, LOC220220, and LOC168991. The *MLL2* gene was expressed in three cases with HBV DNA integrated into exon 3 of *MLL2* and in one case with HBV DNA integrated into intron 3 of *MLL2*. Gene expression analysis suggested that two HCCs with HBV integrated into *MLL2* had similar patterns of gene expression compared with three HCCs with HBV integrated into other loci of human chromosomes.

Conclusions: HBV DNA was integrated at random sites of human DNA, and the *MLL2* gene was one of the targets for integration. Our results suggest that HBV DNA might modulate human genes near integration sites, followed by integration site–specific expression of such genes during hepatocarcinogenesis.

Hepatocellular carcinoma (HCC) is one of the most lethal cancers in the world (1). Epidemiologic data suggest that hepatitis B virus (HBV) is closely related to hepatocarcinogenesis (2). Southern blot analysis has shown that HBV DNA is frequently integrated into the human genome in HCC (3). Such insertional mutagenesis is one of the most important mechanisms for the development of HBV-related HCC. Critical genes adjacent to integrated HBV DNA have been identified by molecular techniques. HBV DNA integration has occurred in an exon of the *retinoic acid receptor B* gene (4), and the *cyclin A2* gene was identified at an early stage of HCC (5). However, these findings have not been reproduced. Woodchuck hepatitis virus DNA, which induces liver tumors in woodchucks, was found to be integrated in c-*myc* or N-*myc* in 30% of liver tumors, most of them exhibiting increased expression of these genes (6). It thus remains unclear which genes serve as targets for HBV DNA integration in human liver.

Recent studies have reported that some modified PCR techniques could effectively detect the cellular DNA sequences adjacent to an integrated retroviral provirus (7, 8). One of these techniques, cassette ligation–mediated PCR, is used to selectively amplify utilized DNA when sequence information on a portion of the gene is available (9, 10). In this study, we used cassette ligation–mediated PCR to identify human genome sequences adjoining integrated HBV DNA in HCC. In three cases, HBV DNA was integrated into the *mixed lineage leukemia 2* (*MLL2*) gene. To investigate changes in gene expression patterns caused by HBV DNA integration, we conducted cDNA microarray expression experiments. We confirmed that HCCs with HBV integrated into MLL2 had characteristic patterns of gene expression, compared with HCCs with HBV integrated into other loci of human chromosomes. Finally, we identified candidate genes whose expression was associated with MLL2.

Materials and Methods

Cell culture. PLC/PRF/5 cells were grown in DMEM supplemented with 10% fetal bovine serum. This cell line was used as a positive control of HBV DNA integration into human genome (11).

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Patients and liver tissue samples. We studied 15 consecutive specimens of HCC resected from patients with serum HB surface antigen (HBsAg) after obtaining informed consent. The patients' clinical profiles are shown in Table 1. We examined the normal liver from four patients without HBsAg who had undergone resection for metastatic liver tumors.

DNA and RNA isolation from surgical specimens. Genomic DNA was extracted from cell lines and tumor tissues by proteinase K digestion followed by phenol/chloroform extraction, as previously described (12). RNA was extracted from 11 tumor specimens and 11 noncancerous tissue specimens by the acid guanidinium thiocyanate/phenol/chloroform method (13). The quantity and quality of RNA extracted from each sample was evaluated by RNA6000 Nano Assay (Agilent Technologies, Palo Alto, CA). We used RNA with the recognized 18S and 28S rRNA from both tumor and noncancerous tissues in the same patient for cDNA array analysis.

Cassette ligation–mediated PCR. Human genomes adjacent to the integrated HBV DNA were cloned by using an *in vitro* LA cloning kit (Takara Bio, Inc., Otsu, Japan) as described previously (9). Briefly, 10 μg of the DNA were digested with *Eco*RI, *Hin*dIII, or *Pst*I and ligated to double-stranded DNA cassettes with compatible ends (Fig. 1A). The cassette-ligated DNA fragments were used as a template for nested PCR with the cassette- and HBV-specific primers. One microliter of the DNA solution was amplified in 40 μL of a reaction buffer containing 10 pmol of the two appropriate primers, four deoxynucleotides each at a concentration of 100 mmol/L, PCR buffer, and 2.5 units of *LATaq* polymerase. The amplifications were carried out in a thermal cycler for 33 cycles (45 seconds at 94°C, 2 minutes at 55°C, 2 minutes at 72°C), with final extension for 10 minutes at 72°C. With 1 μL of the first PCR product, a second PCR was done. The sequences of the cassette-specific primers were 5′-GTACATATTGTCGTTAGAACGCGTAA-TACGACTCA-3′ (outer primer) and 5′-CGTTAGAACGCGTAATAC-GACTCACTATAGGGAGA-3′ (inner primer). The sequences of the HBV-specific primers were 5′-ACTCTACCGTCCCCTTCTTCATCTGCC-GTT-3′ (outer primer) and 5′-CTCTTTACGCGGTCTTTTTGTCTGTG-CCTTC-3′ (inner primer).

Subcloning and sequencing. The PCR products were separated by electrophoresis in 1% agarose gel. We cut out each PCR product from the agarose gel and subcloned the products into TA cloning vector (Invitrogen Corp., Carlsbad, CA). The cloning double-stranded DNA was sequenced by the dideoxy method with fluorescently labeled 2′,3′-dideoxynucleoside 5′-triphosphate with the cassette- or HBV-specific primer. Gel electrophoresis and DNA sequencing were done with a DNA sequencing system (377A, Applied Biosystems, Tokyo, Japan). The detailed protocol was reported previously (12).

Homology analysis. We compared the sequences adjacent to the integrated HBV DNA with the human genome using the GenomeNet (http://www.genome.ad.jp).

Southern blot analysis and reverse transcription-PCR for MLL2. The procedure for Southern blot analysis was as described previously (14). In detail, 10 mg from each sample of DNA was completely digested with *Eco*RI and *Bam*HI. The digested DNA was separated on 1% agarose gels and transferred to Hybond-N+ nylon membranes (Amersham Japan Corp., Tokyo, Japan). The membrane was hybridized to a ^{32}P-labeled part of the *MLL2* gene and washed twice in 0.1× SSC/0.1% SDS. The blots were autoradiographed.

We used the amplified product from patient B91 as a probe. RNA samples were incubated with reverse transcriptase (Life Technologies, Gaithersburg, MD) and 25 pmol of the oligo(dT) primer. Then, 2 μL of the cDNA obtained were amplified in 40 μL of a reaction buffer containing 20 pmol of the two appropriate primers, four deoxynucleotides each at a concentration of 100 mmol/L, PCR buffer, and 2.5 units of human recombinant *Taq* polymerase (Takara Bio). Thirty-five cycles of amplification were done (30 seconds at 95°C, 60 seconds at 55°C, and 90 seconds at 72°C) in a thermal cycler for the first PCR. The MLL2 primers used were 5′-TGTGACGACTGAGGTAGAAG-3′ (forward primer) and 5′-CCTGGGTACTCTGTCTGATC-3′ (reverse primer). We used β-actin primers as an internal control (15).

cDNA microarray. The RNA6000 Nano Assay indicated that five cases were suitable for cDNA microarray analysis. In brief, cases B68 and B96 were HCC with HBV DNA integrated into *MLL2*. In cases B59, B95, and B97, HBV DNA was integrated into other loci. The cDNA microarray contained 3,000 cDNA clones (Takara Bio). Preparation of fluorescent cDNA with a direct labeling approach and cDNA microarray hybridization have been described previously, with tumor samples labeled in red (Cy5) and noncancerous liver samples labeled in green (Cy3).

Data preprocessing and analysis. Subtracting the background signals from the spot signals, we obtained (R, G) type of gene expression data,

Table 1. Patient profiles and HBV DNA integration sites

Case no.	Sex	Age	HBsAg	Anti-HCV	Tumor size (mm)	Tumor differentiation	Background	Chromosomal localization	Sequence accession no.
B13	M	50	+	–	40 × 40	Moderate	CH	1q41	Z98267
B19	F	58	+	–	100 × 80	Moderate	CH	11q24.3	AP003481
B36	M	59	+	–	80 × 80	Poor	LC	16q12	AC007491
B43	F	56	+	–	90 × 90	Poor	LC	2q36.3	AC010899
B52	M	59	+	–	30 × 25	Well	LC	5p15.5	AC016575
B57	M	53	+	–	29 × 25	Poor	CH	19q13.1	AD000671
B59	M	64	+	–	20 × 20	Poor	LC	6p23	AL355336
B68	M	47	+	–	140 × 100	Moderate	LC	19q13.1	AD000671
B80	M	68	+	–	25 × 20	Poor	LC	2p16	AC087073
B84	M	40	+	–	30 × 30	Moderate	CH	14q24-31	AC006536
B95	M	50	+	–	22 × 20	Poor	CH	10p12.1-12.3	AL157831
B96	M	55	+	–	80 × 70	Poor	CH	19q13.1	AD000671
B97	M	57	+	–	20 × 20	Moderate	CH	8q11	AC064807
B99	M	52	+	–	45 × 40	Moderate	LC	1p35	AC023225
B107	M	59	+	–	82 × 97	Moderate	CH	7q21	AL109614

NOTE: +, present; –, absent.
Abbreviations: CH, chronic hepatitis; LC, liver cirrhosis.

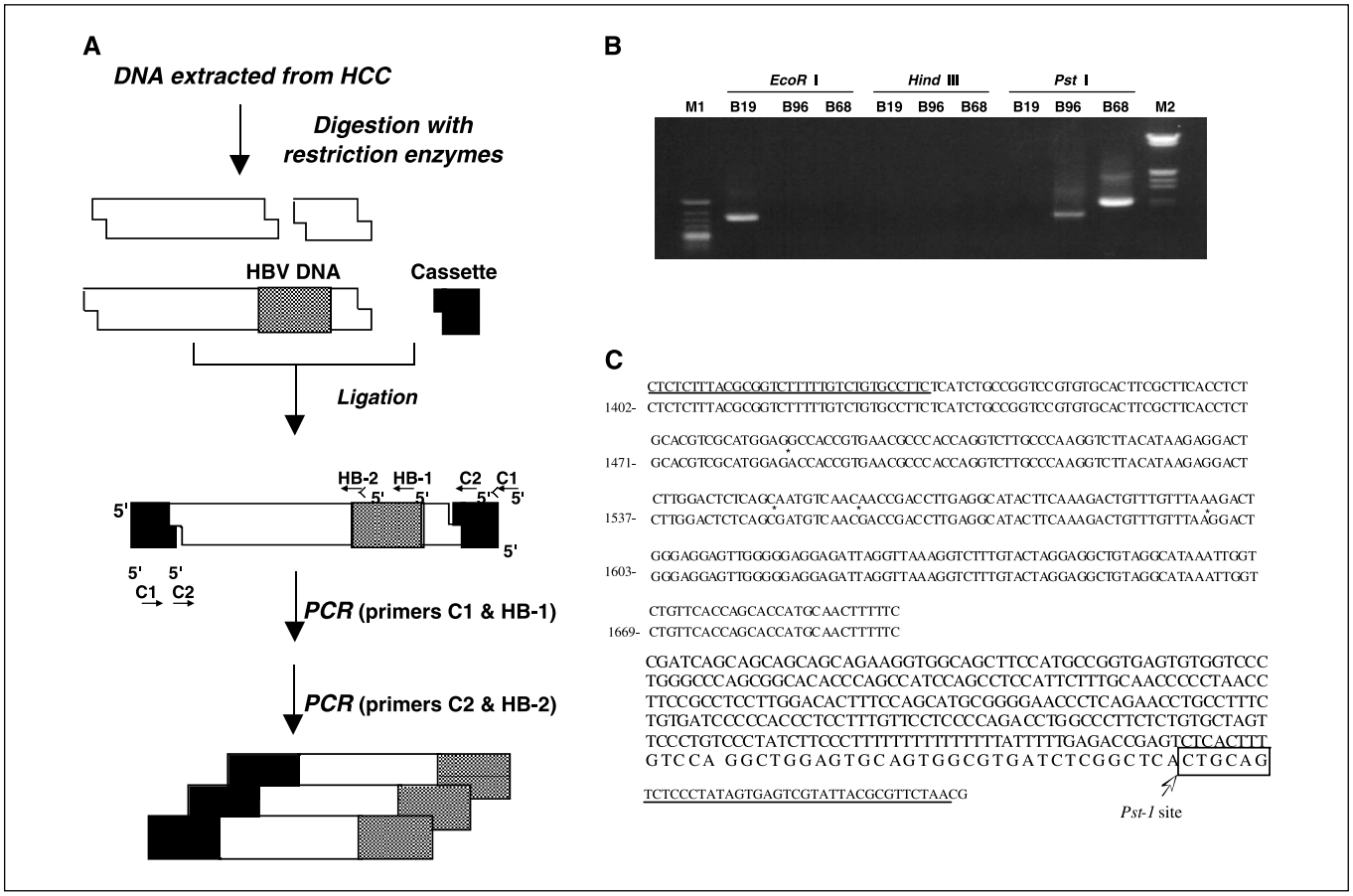

Fig. 1. *A*, schema of cassette ligation – mediated PCR to identify junctions between HBV DNA and human DNA and to sequence the region on either side of the junctions. White bars, human DNA treated with one of three restriction enzymes. Gray bars, HBV DNA. Black bars, cassette DNA. Bars at bottom, amplified PCR products. Small arrows, directions of the primers. After ligation of DNA treated with a restriction enzyme to a DNA cassette, nested PCR was done with two sets of primers. Primers HB-1 and HB-2 were specific for HBV DNA; primers C1 and C2 were specific for the DNA cassette. *B*, electrophoresis of products after the cassette ligation – mediated PCR in 1% agarose gel. In case B19, a 591 bp product was amplified after ligation of the *Eco* RI binding cassette. After ligation of the *Pst* I binding cassette, 650 and 861 bp products were amplified in cases B96 and B68, respectively. M1, size marker, ϕX174/*Hin*cII digestion. M2, size marker, λ/*Hin*dIII, *Eco* RI digestion. *C*, sequence of the subcloning product from case B96. It consists of an HBV DNA sequence (small capital letters) and an *MLL2* sequence (large capital letters). Small capital letters indicate the sequence of HBV DNA (adr; ref. 36). Primers used are shown as an underlined sequence. *, mismatched nucleotides between HBV DNA and the subcloning product.

where R is the signal for Cy5 and G is the signal for Cy3. We computed the logarithm of the expression ratio of Cy5 to Cy3 to evaluate the change in gene expression, and applied a global normalization to the data to correct for bias of the fluorescent dye between Cy3 and Cy5 (16). We used ANOVA to confirm the statistical significance of variability in gene expression between samples. ANOVA uses variances to test the equality of three or more means at one time (17). We also did principal component analysis with varimax rotation to make it easier to interpret the relations between samples. The objectives of principal component analysis are to discover or to reduce the dimensionality of the data set and to identify new meaningful underlying components (17). The varimax rotation is a useful tool for finding more meaningful components (17). All statistical analyses in this study were done with a freely available R statistical software package (http://www.r-project.org).

Ethical considerations. This study protocol complied with the ethical guidelines of the Declaration of Helsinki (1975) and was approved by the Ethics Committee of Osaka City University Graduate School of Medical.

Results

Cassette ligation–mediated PCR. In PLC/PRF/5 cells, DNA fragments were amplified by cassette ligation–mediated PCR. In all 15 HCCs with HBsAg, DNA fragments were amplified. A single PCR product was amplified from each sample obtained from the 15 patients. The size of the DNA product ranged from 581 to 1,200 bp (Fig. 1B). In normal liver without HBsAg, no DNA fragment was amplified.

Sequencing and homology analysis. After subcloning, we determined the sequences of the PCR products. All sequences consisted of an HBV DNA sequence and an unknown sequence. The amplified unknown sequences shared homologies with human DNA. In PLC/PRF/5 cells, HBV DNA was integrated into the human genome at chromosome 13q22. We also confirmed that HBV DNA was integrated into human DNA in all 15 HCCs infected with HBV (Table 1). The locations of the chromosomes with integrated HBV DNA were 1p, 1q, 2p, 2q, 5p, 6p, 7q, 8q, 10p, 11q, 14q, 16q, and 19q. In 10 of the 15 HCCs with integrated HBV DNA, the human sequences adjacent to HBV DNA were thought to be protein-coding regions. These regions were LOC220272 in case B19; ras-responsive element–binding protein 1 in case B59; LOC255345 in case B80; LOC220220 in case B95; calmodulin 1 in case B84; LOC168991 in case 97; FLJ333655 in case B99; and MLL2 in cases B57, B68, and B96. In brief, in case B57,

HBV DNA was integrated into exon 3 of genomic MLL2 (nucleotide 16,656 in chromosome 19-cosmid f24109). HBV DNA was integrated into nucleotides 17,276 and 17,710 of chromosome 19-cosmid f24109 in cases B96 and B68, respectively (Fig. 2A).

Analysis of MLL2 in HCC. The *MLL2* gene, located on chromosome 19q13.1, is related to human leukemia. Therefore, we examined MLL2 in HCC. Southern blot analysis showed amplification of the *MLL2* gene in tumor from patient B68 (Fig. 2B). Reverse transcription-PCR indicated that MLL2 was expressed in HCC and not in noncancerous liver tissue from cases B57, B68, and B96 (Fig. 2C). MLL2 was not expressed in HCC from any other cases.

Analysis of cDNA microarray. We constructed a set of gene expression profiles for five samples in Table 1 (cases B68, B96, B59, B95, and B97) on the basis of the results of cDNA microarray experiments. First, we applied one-way ANOVA between the samples. The results suggested that the expression patterns varied significantly between samples (P = 0.00031). Next, for easier interpretation of the relations between samples, we examined the expression profiles by principal component analysis with varimax rotation. Figure 3 shows a scatter plot of the weights of the first and second principal components (called PC1 and PC2 weights below), which represent the relations between samples. Cases 68 and 96 seem to be located close to each other and far from the other samples, indicating that the gene expression patterns of cases 68 and 96 differ from those of the other cases (cases B59, B95, and B97). This result suggests that the samples of HCC with HBV integrated into MLL2 had characteristic gene expression profiles compared with HCC with HBV integrated into other loci of human chromosomes. Figure 4 shows a scatter plot of the scores of the first and second principal components (called PC1 and PC2 scores below), which represent the relations between the genes used in this study. By contrasting the principal component weights in Fig. 3 with the principal component scores in Fig. 4, low scoring genes in PC1 and high scoring genes in PC2 can be identified as candidates for differentially expressed genes related to cases 68 and 96. We computed the sum of negative PC1 scores and positive PC2 scores, and selected genes in the upper 1%. We picked up the candidate genes possibly involved in the differences in expression profiles between loci to be integrated. These data will be available in the Kanehisa Lab web page (http://web.kuicr.kyoto-u.ac.jp/~yoshi/tamori/expression.html). Information is available from the authors on request.

Discussion

We identified new integration sites of HBV DNA in all 15 cases of HCC associated with HBV infection by cassette ligation–mediated PCR. In the positive control, PLC/PRF/5

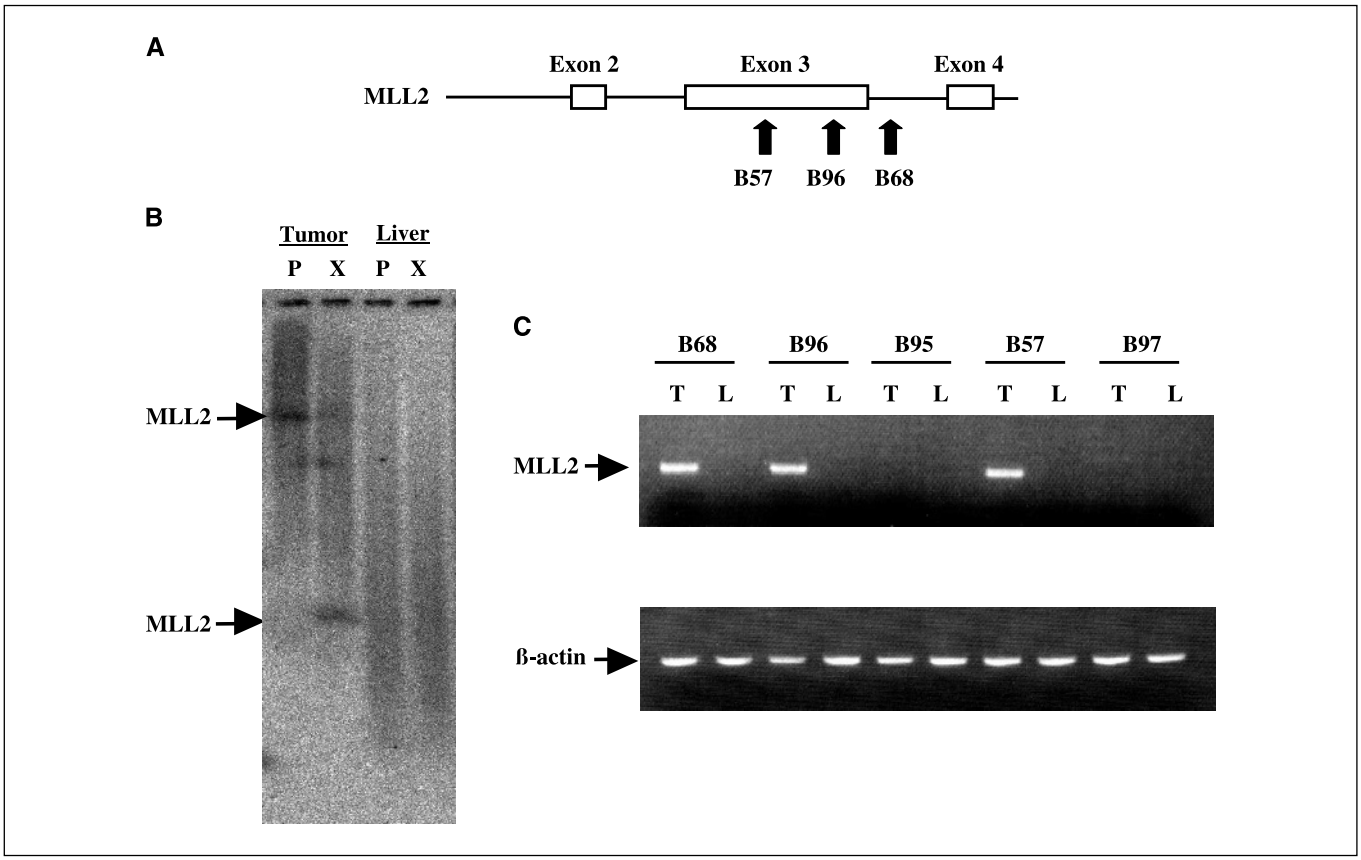

Fig. 2. HBV DNA integrated into genomic MLL2. *A*, schematic alignment of genomic MLL2. In B57 and B96, HBV DNA was integrated into exon 3 of genomic MLL2. In B68, the integration site was at intron 3 of genomic MLL2. *B*, Southern blot analysis with a ^{32}P-labeled MLL2 probe showed an amplified DNA in the tumor from patient B68. P, *Pst*I-digested sample; X, *Xba*-digested sample. *C*, reverse transcription-PCR showed that MLL2 mRNA was expressed only in the tumor with HBV DNA integrated into genomic MLL2. T, tumor; L, noncancerous liver.

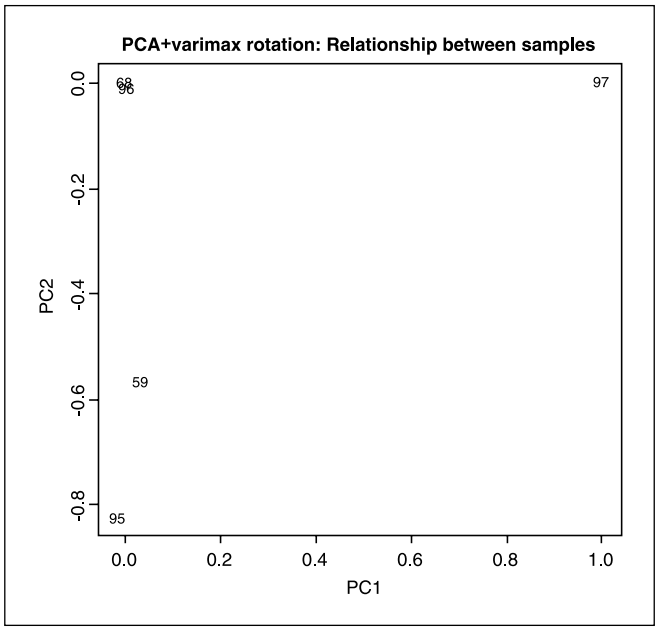

Fig. 3. The scatter plot of the first and second principal component loadings derived from the principal component analysis for the five samples (cases 59, 68, 95, 96, and 97). Cases 68 and 96 were located near each other on the graph.

cells, HBV DNA integration sites were also detected. On the other hand, no HBV DNA was amplified in the negative control, normal liver without HBsAg. We believe that the cassette ligation–mediated PCR worked correctly. We used parts of the *hepatitis B x* sequence as specific primers for two reasons. First, HBV DNA has two direct repeat sequences near the *hepatitis B x* region, and such sequences seem to be involved in DNA integration (18). Second, hepatitis B x antigen has been detected in many specimens of HCC (19, 20). *Alu*-HBV-PCR, one of the most sensitive methods, amplified HBV integrated into human DNA in all HCCs of 33 patients with HBsAg (21, 22). Our results indicate that cassette ligation–mediated PCR and *Alu*-HBV-PCR can similarly amplify HBV DNA integrated into human DNA.

Next, we confirmed that the sequences found in this study had homologies with human sequences. They were not previously reported to be successive sequences to integrated HBV DNA. Prior studies have shown several HBV insertions in chromosomes 8, 11, and 17 (23–25). In the present study, HBV DNA integration was found in chromosome 19q in three tumors. Other tumors had different integration sites. It has been suggested that HBV DNA integrates into DNA in hepatocytes in the early stage after infection (26). Because HBV DNA integrates into random positions of the human genome, all HBV DNA integration does not directly transform hepatocytes. In the present study, the human DNAs located near the integrated HBV DNA were coding proteins in 10 of the 15 HCCs. Among these proteins, ras-responsive element–binding protein 1, calmodulin 1, and MLL2 have been reported to have functions. Ras-responsive element–binding protein 1, the ras-responsive zinc finger transcription factor, modulates Ras and Raf signal transduction in medullary thyroid cancer (27) and down-regulates *p16* promoter (28). Calmodulin 1 is a calcium-modulated protein able to regulate the cell cycle by binding p21 (29). MLL2 is a homologue of the *Drosophila trithorax* gene (30), which is involved in translocations in infantile leukemia and is amplified in some adult myeloid leukemias (31). Available evidence indicates that MLL2 and ras-responsive element–binding protein 1 are related to human carcinogenesis. MLL2 consists of 8.5 kb of cDNA sequences and 37 exons, closely resembling the MLL coding protein (32). *MLL2* is amplified in some pancreatic carcinoma cell lines and glioblastoma cell lines (33). To our knowledge, expression of *MLL2* in HCC has not been reported previously. This is the first study to report that the *MLL2* gene was amplified and expressed in HCC with HBV DNA integrated into genomic *MLL2*.

Next, cDNA microarray experiments showed that gene expression profiles distinctly differed between HCC with HBV DNA integrated into *MLL2* and HCC without such integration. Interestingly, expression levels of the genes contributing to the distinction of these two groups were reduced. In contrast, the expression levels of 343 genes increased in both groups for all five samples. Apart from the integration site in the human genome, these genes might be universal families of genes whose expression levels increase with hepatitis B infection. It is unclear why the expression levels of discriminative genes for HCC with HBV DNA integrated into *MLL2* decreased. The expression of these genes might have been altered by integration of HBV DNA into *MLL2*. Our results suggested that *MLL2* was one of the targets for HBV DNA integration and indicate that MLL2 function was critical for hepatocarcinogenesis in these patients.

Recent studies have reported the insertion of HBV DNA into *SERCA-1* or *human telomerase reverse transcriptase* (34, 35) and suggested that the amplification of genes plays an important role in carcinogenesis. Such genetic changes do not commonly apply to all HCCs, as shown by our results. We believe that HBV DNA is randomly integrated into human DNA. When integration hits genes related to cell growth or death, hepatocarcinogenesis by HBV would begin. Since the time of rough determination of the human genome sequence,

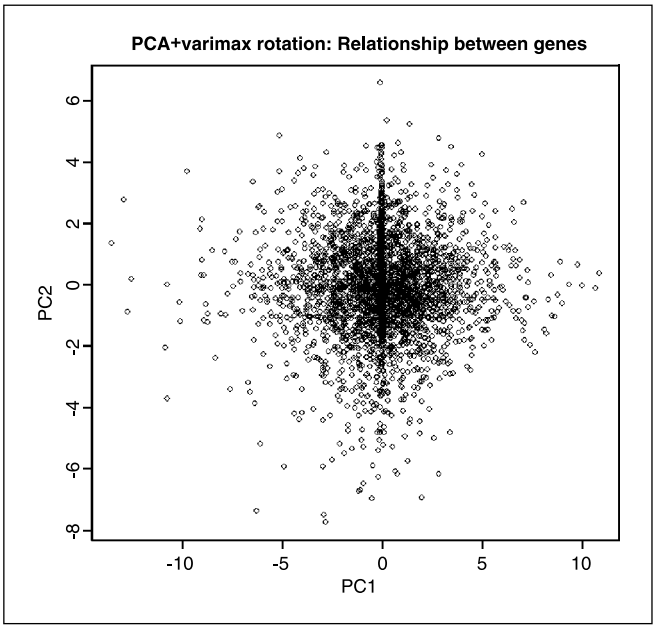

Fig. 4. The scatter plot of the first and second principal component scores derived from the principal component analysis for the 3,055 genes used in cDNA microarray experiments. In the figure, each circle corresponds to one gene.

increasing genetic information has become available. In the near future, we will be able to explore the functions of genes at sites of HBV DNA integration and identify individual pathways leading to HCC in patients with HBV.

In conclusion, we sequenced human DNA adjacent to integrated HBV DNA in 15 cases of HCC by cassette ligation–mediated PCR. HBV DNA integration might alter human gene expression in HCC. Our results suggest that integration of HBV DNA might be an important step in hepatocarcinogenesis and modulate gene expression. To further elucidate the relation between HBV integration and hepatocarcinogenesis, future studies should examine the function of human genes adjacent to integrated HBV DNA in more cases of HCC.

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