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Identification and extensive analysis of inverted-duplicated HBV integration in a human hepatocellular carcinoma cell line

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Hepatitis B virus (HBV) DNA is often integrated into hepatocellular carcinoma (HCC). Although the relationship between HBV integration and HCC development has been widely studied, the role of HBV integration in HCC development is still not completely understood. In the present study, we constructed a pooled BAC library of 9 established cell lines derived from HCC patients with HBV infections. By amplifying viral genes and superpooling of BAC clones, we identified 2 clones harboring integrated HBV DNA. Screening of host-virus junctions by repeated sequencing revealed an HBV DNA integration site on chromosome 11q13 in the SNU-886 cell line. The structure and rearrangement of integrated HBV DNA were extensively analyzed. An inverted duplicated structure, with fusion of at least 2 HBV DNA molecules in opposite orientations, was identified in the region. The gene expression of cancer-related genes increased near the viral integration site in HCC cell line SNU-886. [BMB Reports 2012; 45(6): 365-370]

INTRODUCTION

The role of hepatitis B virus (HBV) integration in the development of hepatocellular carcinoma (HCC) is still not completely understood. Although HBV integration does not seem to be absolutely required for HCC development, HBV integration has been identified in most cases of HBV-related HCC and usually precedes HCC development (1-3). There are 2 possible major carcinogenic effects of HBV DNA integration on HCC development: one is the *trans*-activating function of viral proteins, such as HBx and PreS2, and the other is the *cis*-activating function of viral DNA (1, 4). The HBV insertion within or close to a tumor-related gene may subsequently alter expression of the gene and cause HCC by interrupting open reading frames or by activating genomic transcription through viral enhancer activity (5-7). For example, inserting an HBV enhancer into the hTERT promoter region causes the *cis*-activation of the hTERT gene in HCC cells (8). Integration of HBV DNA may rearrange the viral genome and/or the host chromosome at the integration site and may alter the expression and/or function of nearby cellular genes (5). In addition to *cis*- and *trans*-activating mechanisms, HBV DNA integration alters gene expression by promoting genetic instability, and/or by altering expression of regulatory genes, such as microRNAs (9, 10). Although the relationship between HBV integration and HCC development has widely been studied, only a limited number of studies have reported an association between HBV DNA integration and dysregulation of nearby cellular genes in HCC cells (11-13). For many years, it has been thought that HBV DNA integration events are random with regard to their sites within the human genome. However, when the relationship between fragile sites and virus integration events are compared, HBV DNA is found to integrate within or near many of these regions (9). Although fragile sites are stable in normal cells, they display gaps and breaks in cells under stress where completion of DNA replication is delayed (14).

In the present study, we constructed a pooled BAC library with 9 established cell lines derived from HCC patients and screened for HBV integration sites using the pooled BAC library. HBV integration was identified and characterized on chromosome 11q13 region, a fragile site. The mRNA expression of cellular genes adjacent to the HBV integration site was measured and compared with other cell lines.

RESULTS

BAC library construction

We constructed a pooled BAC library of 9 established HCC cell lines (SNU-354, SNU-368, SNU-387, SNU-398, SNU-423, SNU-449, SNU-475, SNU-761 and SNU-886) derived from Korean patients with liver cancer. HBV infections in the 9 HCC cell lines were confirmed by PCR amplification with pri-

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mers designed to target HBV genes (C, P, S, and X) (Fig. 1). The genomic DNAs of the 9 cell lines were partially digested with a restriction enzyme (*Hind*III) and ligated with the pBAC-lac vector. A total of 50,688 BAC clones were obtained and prepared on 528 96-well plates. PCR amplification of viral genes and superpooling of BAC clones were employed to rapidly screen clones harboring HBV DNA. From this screening, 2 BAC clones (HBL-0089_H7 and HBL-0089_H8) were identified. The direct end-sequencing of these BAC clones showed that these clones contained approximately 40 kb and 68 kb of cellular DNA from the 14q21 and 11q13 chromosomal region, respectively.

HBV DNA integration

To determine the exact position of HBV DNA integration in BAC clones, we screened the host-virus junction by sequence analysis with primers designed from HBV DNA sequences.

One of the host-virus junctions in the HBL-0089_H8 clone was located between 2 fibroblast growth factor (*FGF*) genes on the chromosome 11q13 region, which was approximately 35 kb upstream of *FGF19* and 33 kb downstream of *FGF4* (Fig. 2). However, we failed to find a second host-virus junction in HBL-0089_H8 and HBL-0089_H7 clones. The origin of the HBV DNA in the HBL-0089_H8 clone was determined by amplifying the host-virus junction sequence in the 9 HCC cell lines. The host-virus junction of the HBL-0089_H8 was amplified only in the SNU-886 cell line (Fig. 2C). Additional analyses of the integration site were performed by direct sequencing and long-range PCR with primers designed from the cloned HBV integrated sequence fragments and chromosomal sequences of the host-virus junction in an attempt to characterize the entire integrated HBV sequence. Using this strategy, a 1.9 kb PCR fragment was amplified and sequenced. Analysis of the PCR product containing the host-virus junction sequence revealed 1,537 bp of HBV sequence (98% identity match to position 1,629-3,165 of the HBV genome) joined to 331 bp of human DNA corresponding to the upstream intergenic region of *FGF19* (position 69,554,259-69,554,589 of human reference genome, build 37.3) (Fig. 2B and D).

The virus-virus junction was also identified by repeated sequencing and confirmed by PCR amplification of the junction sequences with primers designed to identify the HBV rearrangement (Supplemental Table 1). The integrated HBV DNA had an inverted duplicated structure, with fusion of at least 2 HBV DNA molecules in the opposite orientation (Fig. 3A). To verify whether this inverted duplication occurs in the

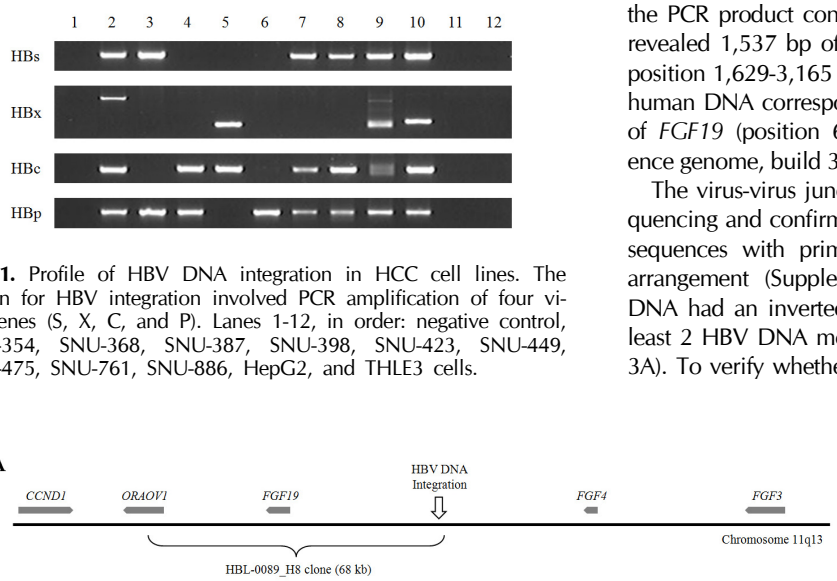

Fig. 1. Profile of HBV DNA integration in HCC cell lines. The screen for HBV integration involved PCR amplification of four viral genes (S, X, C, and P). Lanes 1-12, in order: negative control, SNU-354, SNU-368, SNU-387, SNU-398, SNU-423, SNU-449, SNU-475, SNU-761, SNU-886, HepG2, and THLE3 cells.

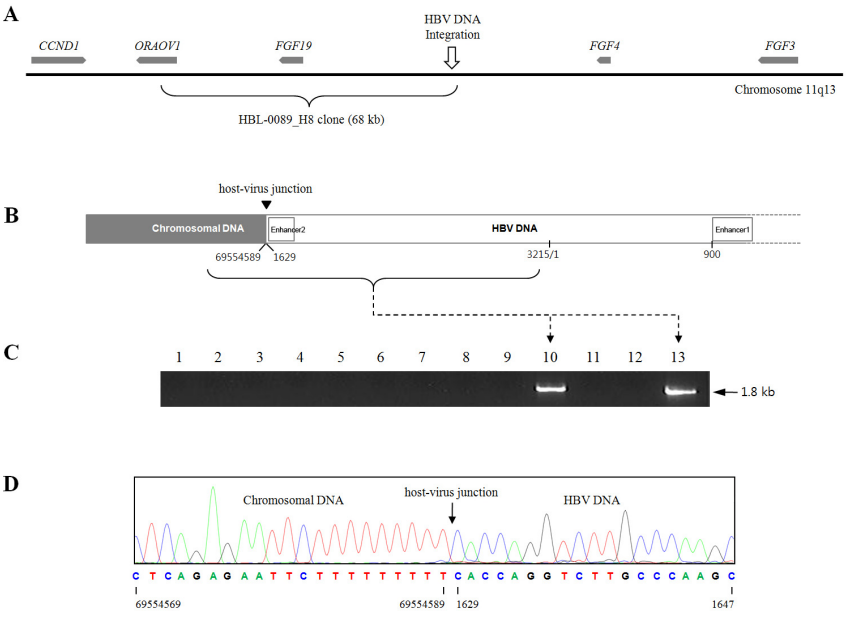

Fig. 2. HBV DNA integration. (A) The map shows the genomic structure of chromosome 11q13 region. Block arrow indicates the HBV DNA integration site between two FGF genes. The HBL-0089_H8 clone contains approximately 68 kb host chromosome including the *FGF19* gene. (B) The diagram is a schematic representation of a host-virus junction in the HBL-0089_H8 clone. (C) Amplification of the host-virus junction. Lanes 1-13, in order: negative control, SNU-354, SNU-368, SNU-387, SNU-398, SNU-423, SNU-449, SNU-475, SNU-761, SNU-886, HepG2, THLE3 cells, and HBL-0089_H8 clone. (D) The PCR product generated from the host-virus junction in SNU-886 was sequenced to determine the identity of host-virus fusion sequences.

SNU-886 cell line or only in the HBL-0089_H8 clone, we amplified the 0.8 kb region of the virus-virus junction by PCR. The virus-virus junction was amplified in both the HBL-0089_H8 clone and the SNU-886 cell line, but not in the other human liver-derived cell lines we tested (SNU-449, HepG2 and THLE3) (Fig. 3B). Sequence analysis showed that an HBV DNA fragment with the truncated S gene (nucleotide 1-501) was attached to another HBV DNA fragment with the truncated P gene (nucleotide 550-1260) (Fig. 3C).

In addition, sequencing analysis revealed multiple types of alterations in the HBV DNA sequences, including deletions and inversions (data not shown). However, HBV enhancer I and II sequences were relatively well retained in the HBV DNA integrant (Fig. 2B and 3B). The integrated HBV sequence in the SNU-886 cell line was genotype C, with the highest homology in GenBank to the HBV strain *adr*, sequence accession number GQ475354.1 (15, 16).

Gene expression at the HBV integration site

We investigated the possible cis-activating effect of HBV DNA integration to the expression of adjacent cellular genes. Real-time RT-PCR was performed to evaluate the levels of cyclin D1 (*CCND1*), oral cancer expressed 1 (*ORAOV1*), *FGF19*, and *FGF4* expression in the SNU-886 and SNU-449 cell lines. The HepG2 and THLE-3 cell lines were used as negative controls because no HBV integrants were found in these cells. The levels of *FGF19* mRNA expression were markedly increased in the SNU-886 cell line. *CCND1* and *ORAOV1* gene expression were also increased significantly in the SNU-886 cell line compared to the other cell lines tested (Fig. 4). However, we could not measure *FGF4* gene expression in the SNU-886 cell line because the level was below the limit of detection in any of the HCC cell lines tested (data not shown).

DISCUSSION

In the present study, we constructed a pooled BAC library from 9 human HCC cell lines and identified 2 HBV integration loci at chromosomes 11 and 14. Because 1.6-2.7 million clones with an average insert size of 100 kb are needed to sufficiently cover the entire genome of 9 diploid cell lines, we could only identify 2 clones, including HBV DNA from a total

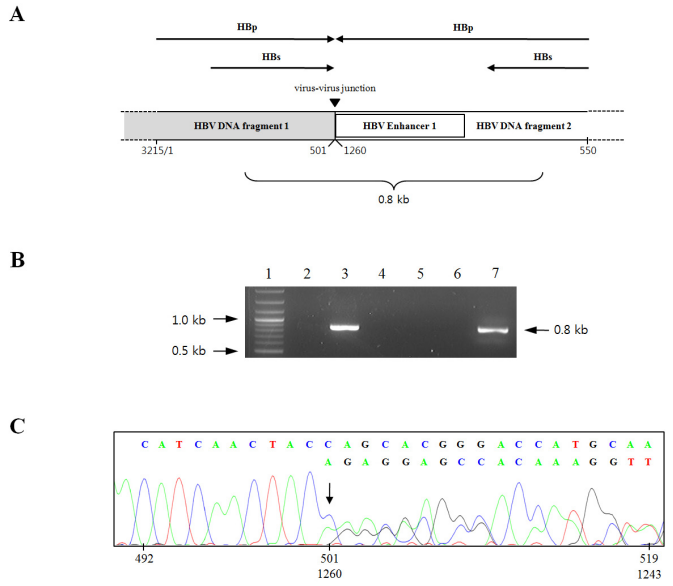

Fig. 3. Identification of HBV DNA rearrangement. (A) The diagram is a schematic representation of the inverted duplication of the HBV DNA integrant. Viral genes near the virus-virus junction are represented by arrows. The numbers indicate the nucleotide positions in HBV genome. (B) Amplification of the virus-virus junction. Lanes 1-7, in order: DNA ladder, negative control, SNU-886, SNU-449, HepG2, THLE3 cells and HBL-0089_H8 clone. (C) The PCR product from the rearranged HBV genome in Fig. 3A-B was sequenced to identify the inverted duplicated structure. The arrow indicates the point of virus-virus junction.

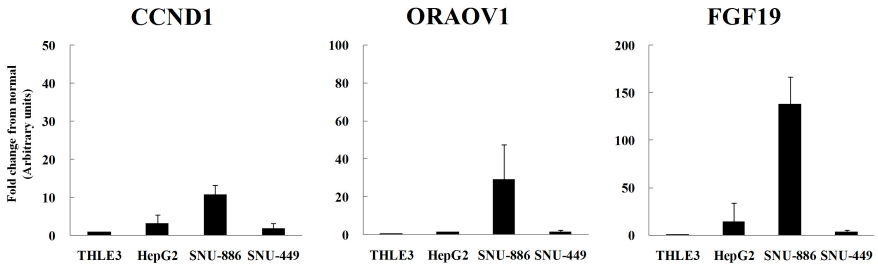

Fig. 4. Gene expression profile of the HBV DNA integration site. Relative mRNA expressions of *CCND1*, *ORAOV1* and *FGF19* were determined by real-time RT-PCR using *GAPDH* expression as the control. The results shown are the mean and standard error of the 3 independent measurements. *FGF19* expression was significantly elevated in SNU-886 cells.

of 50,688 clones. Due to rearrangements and deletions in the viral and host genomic DNA that frequently occur at the viral integration site, we identified only one of the host-virus junctions at the HBV integration site (Fig. 2B). Since it is often difficult to identify the remaining integrated HBV sequences and the second host-virus junction, many studies have provided information on only one of the two host-virus junctions at the integration site (1). It has been speculated that integrated HBV DNA acts as a hot spot for producing an inverted duplicated structure, which may cause amplification of the surrounding regions and induce overexpression of some oncogenes. We also found inverted duplication and partial deletions of integrated HBV DNA (Fig. 3A). The virus-virus junction of inverted duplicated HBV DNA was confirmed by PCR amplification (Fig. 3B) and by direct sequencing (Fig. 3C) of this fusion site. However, the genomic amplification of this region was not defined in our study.

Although the HBV integration site has widely been screened and identified in HCCs, only a limited number of cases have reported viral integration on chromosome 11q13 (17-19). Hatada *et al*. reported HBV integration on chromosome 11q13 by co-amplification of HBV DNA and the *FGF4* gene (17). They also described the inverted duplication of an HBV DNA integrant. However, the effect of viral integration on host gene expression was not described. The 11q13 amplicon containing *CCND1*, cortactin (*CTTN*), and FGF genes is one of the most frequent amplification events in human tumors and is well characterized (20-22). *FGF19* and *CCND1* are invariably coamplified in HCCs, leading to an increase in expression of both genes. *FGF4* and *FGF3* are also frequently coamplified with *CCND1*. However, *CCND1* and *FGF19* are often amplified in the absence of coamplification with these 2 other FGF genes (23). Furthermore, although amplification results in significant increases in *CCND1* and *FGF19* expression in HCC, amplification of *FGF4* and *FGF3* does not correlate with increased gene expression. To examine the expression of these cancer-related genes on chromosome 11q13, we measured mRNA expression levels by real-time RT-PCR. The results showed that *FGF19*, *CCND1* and *ORAOV1* were overexpressed in SNU-886 cells (Fig. 4). *FGF19* plays an important role in mitogenesis, angiogenesis, differentiation, metabolic regulation, tissue repair and oncogenesis (24). Several groups have also reported an association between *FGF19* and HCC. Nicholes *et al*. observed the development of liver tumors in *FGF19* transgenic mice and suggested that *FGF19* acts directly as an HCC tumor promoter (25). Inactivating *FGF19* and/or *FGFR4* reduces tumor growth by deregulating beta-catenin signals (26). Recently, Miura *et al*. reported that the introduction of *FGF19* siRNA can reduce proliferation and increase apoptosis in HCC cell lines. Inversely, recombinant *FGF19* can induce proliferation, stimulate invasion and inhibit apoptosis in HCC cell lines (27). They also reported a significant correlation between *FGF19* expression and the pathological stage and suggested that *FGF19* is an independent prognostic factor for patient survival. Overexpression of *CCND1* and *ORAOV1* has been described in a variety of human cancers (28-30) and is also associated with poor prognosis of HCC (31). However, with the exception of the SNU-886 cell line, the overexpression of *CCND1* and *ORAOV1* was not observed in other hepatoblastoma (HepG2) and HCC (SNU-449) cell lines (Fig. 4). In the SNU-886 cell line, both enhancer I and II sequences were well retained in integrated HBV DNA (Fig. 2B and 3B). While there has been some disagreement regarding the transactivating potential of these enhancers, evidence from a number of groups suggests that the HBV enhancers are capable of *trans*-activating cellular genes up to 100 kb from the point of integration (1, 8, 32). Taken together, we propose the following models for gene activation on the chromosome 11q13 region in SNU-886 cells. Firstly, viral integration induces genetic changes and activation of gene expression at the integration site without gene amplification. Secondly, viral DNA induces gene amplification, causing overexpression during integration and rearrangement. Lastly, gene activation is related to gene amplification, regardless of viral integration. Further studies, including screening of regional amplification at chromosome 11q13 in a larger sample of HCCs are needed to clarify the effect of HBV integration in SNU-886 cells. The pooled BAC library of HCC cell lines may be useful for studying genomic amplification and rearrangement in HCC cells.

MATERIALS AND METHODS

Cell lines

Nine HCC cell lines were used to construct a pooled BAC library (SNU-354, SNU-368, SNU-387, SNU-398, SNU-423, SNU-449, SNU-475, SNU-761, and SNU-886). These cell lines were derived from Korean HCC patients infected with HBV (33, 34). HCC cell lines were obtained from the Korea Cell Line Bank (Seoul, South Korea) and cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY). HepG2 and THLE-3 cells were obtained from the American Type Culture Collection (Manassas, VA), and cultured in phenol red-controlled Eagle's Minimum Essential Medium and Bronchial Epithelial Cell Growth Medium, respectively.

BAC library construction

Genomic DNAs of nine HCC cell lines were extracted from HCC cells embedded in low-melting-point agarose plugs (5×10^6 cells/plug) and pooled. These pooled DNAs were digested separately with restriction enzyme *Hind*III and electrophoresed to isolate 50-150 kb DNA fragments by pulse-field gel electrophoresis, CHEF DRIII (Bio-Rad Laboratories, Hercules, CA). Then, DNA fragments were ligated with the pBAC-lac vector and transformed into *E. coli* DH10B cells. White colonies of BAC clones with human DNA insert were selected, cultured and stored in 96-well plates.

Screening of BAC clone harboring HBV DNA sequences

The detailed protocols for rapid screening of BAC clone harboring specific DNA sequences have been described in the previous report by Asakawa *et al*. (35). Briefly, 44 superpools of DNA were prepared from every 12 plates, and 27 DNA pools were prepared from four-dimensionally assigned BAC clone pools. The first 44 PCR assays identify a particular superpool and the second 27 PCR assays detect the particular BAC clone (s). Primers for PCR screening were designed to target HBV DNA sequences (Supplementary Table 1). The insert size of the cellular DNA was estimated by end-sequencing of multiple cloning sites in the pBAC-lac vector. The HCC cell line, a source of HBV DNA sequence in a BAC clone, was determined by PCR amplification with primers to target specific host-virus junction.

Sequence analysis

The chromosomal position of HBV DNA integration site was determined by identification of host-virus junction using direct sequencing. The structural variation and rearrangement of viral and host DNA were also detected by PCR amplification and sequence analysis. Primers were designed to amplify four HBV genes (C, P, S, and X), host-virus junction, and virus-virus junction using the human reference sequence (NT_167190) and a complete HBV genome sequence (D50519.1). Primer sequences are available in Supplementary Table 1. Direct sequencing was performed with a BigDye Terminator Sequencing Kit and an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols.

Measurement of mRNA expression

The levels of mRNA expression of cellular genes near the HBV integration site were measured by real-time RT-PCR. Total RNA of each cell line was prepared using an Easy Spin Total RNA Extraction kit (Intron Biotechnology, Seoul, South Korea) according to the manufacturer's instructions. After cDNA synthesis, TaqMan-based assays were performed with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's protocols. Ready-to-use primer and probe sets predesigned by Applied Biosystems were used (Hs00277039_M1 for *CCND1*, Hs00411598_M1 for *ORAOV1*, Hs00391591_M1 for *FGF19*, Hs00173564_M1 for *FGF4*, and Hs99999905_M1 for *GAPDH*). *GAPDH* was used as an internal control gene for normalization. The relative and quantitative mRNA expression was calculated using the comparative C_T method (36).

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