TRANSFORMING GENES IN HUMAN HEPATOMAS DETECTED BY A TUMORIGENICITY ASSAY

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Two transforming sequences in human hepatomas were detected by means of a tumorigenicity assay in nude mice using transfected NIH3T3 cells. Through hybridization with known oncogene probes, the transforming gene in one hepatoma was found to be the human $hst$ gene. The transforming sequence in the other hepatoma showed no sequence homology with any of the oncogenes examined.

Key words: Hepatoma — Transforming gene — $hst$

Cellular transforming genes were detected in a wide variety of human tumors and tumor cell lines in a DNA transfection experiment using mouse NIH3T3 cells.1) The majority of the transforming genes identified in this manner belong to the ras gene family.2-5) Hepatomas are very common in Asia and Africa, and the hepatitis B virus (HBV) is known to be associated with some cases of hepatomas. HBV itself does not possess transforming genes, suggesting that cellular transforming genes are involved in hepatoma induction. However, only a few transforming genes have been detected in human hepatomas.6,7) We searched for transforming genes in 20 hepatomas by means of the DNA transfection assay, but in vain.

Tumorigenicity assay of DNA-transfected NIH3T3 cells in nude mice was shown to be useful for detecting weak transforming genes.8,9) We report here the use of the tumorigenicity assay for two human hepatomas and the detection of two non-ras transforming genes.

High-molecular-weight cellular DNAs were extracted from the two human hepatomas. They were a primary tumor, 84-3 (type I according to the Edmondson classification9)), and a TMH-1 tumor (Edmondson type II-III), which was maintained in nude mice. HBV surface antigens in the patients' serum were positive for the TMH-1 tumor and negative for the 84-3 tumor. These tumor DNAs did not induce foci of morphologically altered NIH3T3 cells (data not shown). The tumorigenicity assay was performed as described previously.9) Briefly, cellular DNA (30 μg) and pSV2neo (1 μg) were precipitated with calcium phosphate into 10-cm culture dishes seeded 24 hr earlier with $1 \times 10^6$ NIH3T3 cells in 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% calf serum. After incubation for 12–15 hr, the precipitate was removed and replaced with fresh medium. After an additional 24 hr, the cells in each dish were trypsinized and then seeded into three 10-cm dishes containing fresh medium plus 0.4 mg/ml of the antibiotic, G418. After 12–15 days of growth in the selective medium, the cells were trypsinized and pooled. Approximately $5 \times 10^6$ cells from the equivalent of three dishes were injected subcutaneously into BALB/c nude mice. As a positive control, one set of mice was inoculated with NIH3T3 cells transfected with genomic DNA from B2-2, an NIH 3T3 transformant containing the v-H-ras oncogene. As a negative control, one set of mice was inoculated with cells exposed to salmon sperm DNA.

Tumors arose after one week in all the positive control mice. In the first cycle experiment, the 84-3 hepatoma DNA induced tumors in two mice about 60 days after inoculation (Table I). Tumors arose in three out of four mice with the TMH-1 hepatoma DNA about 65 days after inoculation. There was a low tumor incidence in the negative control group.
HEPATOMA TRANSFORMING GENES

To confirm that the tumors were induced by the human DNAs, the tumor DNAs were subjected to Southern blot analysis using a probe specific for human ‘Alu’ repetitive sequences. Numerous bands were seen in most first cycle tumors induced by the 84-3 and TMH-1 tumor DNAs (Fig. 1, lanes 1 and 3). The Alu-negative tumor may reflect a background of tumor induction, since there was a low tumor incidence when testing normal DNA. The Alu-positive primary tumors with the 84-3 and TMH-1 DNAs were designated as S151 and S150, respectively.

The second cycle tumorigenicity assay was performed with the S151 and S150 DNAs. Alu-positive tumors again appeared in most experiments (Table I). Then, the Alu-positive secondary tumor DNAs were used for the third cycle tumorigenicity assay. All the TMH-1-derived tertiary tumor DNAs retained the two Alu-related bands, 15 and 4 kbp, after digestion with EcoRI (Fig. 1, lane 4), implying that these two fragments are linked to the TMH-1 tumorigenic sequences. The Alu-containing band pattern of the 84-3 DNA-derived tertiary tumor DNA (Fig. 1, lane 2) was different from that of the TMH-1-derived tertiary tumor DNA with EcoRI (Fig. 1, lane 4). After digestion with BamHI, each tumor DNA also showed a different Alu-containing band pattern (data not shown). These results indicate that the two tumorigenic sequences were different.

To determine if there was any relationship between the two hepatoma tumorigenic sequences and any known oncogene, the nude mouse tumor DNAs were subjected to molecular hybridization with 14 oncogene probes: K-ras, H-ras, N-ras, erbB, erbB2/neu, fos, myb, c-myc, N-myc, raf, ros, yes, hst and lca. Only with the hst cDNA probe did the EcoRI-digested, 84-3-derived tumor DNAs exhibit additional hybridizing bands not seen in mouse DNA (Fig. 2). EcoRI-digested human DNA showed four bands of H1 (8.4 kbp), H2 (6.0 kbp), H3 (2.8 kbp) and H4 (0.8 kbp) (Fig. 2, lane 1). The H4 band was faint because of its small size. The three bands (H2-H4) are derived from the hst gene, whereas the H1 fragment is derived from the other gene related to the hst gene (M. Terada et al., personal communication). Three hst-related bands (M1-M3) were observed for the

Table I. Tumorigenicity Assay of the 84-3 and TMH-1 Tumor DNAs

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Expt</th>
<th>Tumor incidence</th>
<th>Alu(+)</th>
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<tr>
<td>First</td>
<td>1</td>
<td>2/2</td>
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<tr>
<td></td>
<td>2</td>
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<td>Second</td>
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<td></td>
<td>2</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Third</td>
<td>1</td>
<td>3/4</td>
<td>2/3</td>
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<tr>
<td></td>
<td>2</td>
<td>2/4</td>
<td>1/2</td>
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a) Number of tumors per number of inoculated mice.
b) Number of Alu sequence-positive tumors per number of tumors examined.

Fig. 1. Detection of human DNA sequences in mouse tumor DNAs. DNAs (20 μg) from mouse tumors were digested with EcoRI. The digested DNAs were electrophoresed in 1% agarose gels which were blotted onto nitrocellulose filters as described by Southern. The filter was hybridized with the nick-translated ‘Alu’ sequence as a probe in 50% formamide/0.75M sodium chloride/0.075 M sodium citrate at 42° for 24 hr. DNAs were obtained from 84-3-derived primary (lane 1) and tertiary (lane 2) tumors and TMH-1-derived primary (lane 3) and tertiary (lane 4) tumors. Coelectrophoresed DNA fragments of HindIII-digested λcI857 DNA served as standards. kbp, kilobase pairs.
mouse DNA after digestion with EcoRI (Fig. 2, lane 2). The 84-3-derived primary (Fig. 2, lane 4) and tertiary tumor DNAs (Fig. 2, lane 5) showed two extra fragments, H3 and H4, besides the mouse endogenous bands and a dense M3 band. The dense M3 band may contain the H2-derived sequences in addition to the mouse M3 fragment, since the H2 band could not be seen in the tumor DNAs. These data indicate that the 84-3 tumorigenic gene is the human hst gene. The original 84-3 hepatoma DNA did not show any abnormality of the hst gene (Fig. 2, lane 3).

The TMH-1-derived tumor DNA did not exhibit any extra bands with the 14 oncogene probes including the hst gene (Fig. 2, lane 6). The EcoRI-digested Alu-containing band pattern of the TMH-1-derived tumor DNA seems to be different from that of the transformants with Blym,14) dbl,15) mel,16) met,17) ret,18) trk19) and mcf-2.8) Thus, it is likely that the TMH-1 tumorigenic gene is a novel transforming gene or one of the genes for which we have not examined the homology yet.

We next examined whether or not the HBV genome was present in the mouse tumor DNAs to determine the association, if any, of the HBV genome and the tumorigenic activity. The HBV genome was not found in any of the mouse tumor DNAs (Fig. 3, lanes 3 and 4). The original TMH-1 hepatoma DNA pos-

![Fig. 2. Identification of the transforming genes. The DNAs (20 µg) were digested with EcoRI and then hybridized with the AvaII 0.6 kbp fragment containing most of the open reading frame 1 of the human hst cDNA13) as described in the legend to Fig. 1. DNAs were obtained from human normal M413 fibroblasts (lane 1), mouse NIH3T3 cells (lane 2), the 84-3 hepatoma (lane 3) the 84-3-derived primary (lane 4) and tertiary (lane 5) tumors, and a representative TMH-1-derived tumor (lane 6). The numbers (kbp) on the left denote the molecular weight markers. The arrows on the right indicate human (H1–H4) and mouse (M1–M3) hst-related fragments.](image)

![Fig. 3. Detection of the HBV sequences in the tumors. The DNAs (20 µg) were digested with HindIII and then hybridized with the entire 3.2 kbp HBV genome as described in the legend to Fig. 1. DNAs were obtained from the human 84-3 (lane 1) and TMH-1 (lane 2) hepatomas and the representative 84-3-derived (lane 3) and TMH-1-derived (lane 4) mouse tumors. The numbers (kbp) on the left denote the molecular weight markers.](image)
sessed multiple HBV genomes (Fig. 3, lane 2). These results suggest that the HBV genome was not involved in the tumorigenic activity.

Transforming genes in human hepatomas have rarely been detected in DNA transfection experiments. Only the activated N-ras\(^6\) and \(lca\) genes\(^7\) have been reported. We detected two transforming sequences in two human hepatomas by means of the tumorigenic assay. These two hepatoma DNAs were found not to induce foci in the NIH3T3 assay. No foci were seen even with the primary and secondary mouse tumor DNAs (data not shown). Thus, the tumorigenic assay is highly sensitive as to the detection of transforming genes in human hepatomas.

The TMH-1 hepatoma contained multiple copies of HBV. However, the TMH-1 DNA-derived mouse tumor DNA did not contain any HBV genome. It seemed likely that the HBV genome was not associated with the tumorigenic sequences, for example, as a promoter in this system.

The 84-3 transforming gene was found to be the human \(hst\) gene. The \(hst\) gene was first detected as a transforming gene in a human stomach cancer and did not show any homology with the known oncogenes\(^{12}\). After digestion with EcoRI, the 84-3 DNA-derived mouse tumor showed the rearranged human \(hst\)-derived band, while the 84-3 human hepatoma DNA exhibited normal \(hst\) bands. The altered band size of the \(hst\) gene would result from DNA rearrangement during or subsequent to transfection. It remains to be determined whether or not the rearrangement was a causative event as to the activation of the \(hst\) gene.

The \(hst\) genes from other tissues have been shown to induce foci in culture\(^{12,13}\), while the 84-3 \(hst\) gene did not. It has been reported that the amplified normal N-ras gene could induce tumors in the tumorigenicity assay.\(^8\) Thus, the amplified \(hst\) gene may contribute to tumor induction in the tumorigenicity assay, even when the \(hst\) gene does not contain structural mutations.

The TMH-1 transforming sequence did not hybridize with 14 oncogene probes. The molecular cloning of the TMH-1 transforming sequence is in progress. The restriction mapping and nucleotide sequence analyses of the TMH-1 transforming gene will reveal its nature and similarity, if any, to other oncogenes.

While this paper was in preparation, Koda et al\(^{20}\) reported that the \(hst\) gene was found in NIH3T3 cells transformed with DNA from three stomach cancers and a colon cancer.

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