Identification of the Gene for a Novel Liver-Related Putative Tumor Suppressor at a High-Frequency Loss of Heterozygosity Region of Chromosome 8p23 in Human Hepatocellular Carcinoma

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Human chromosome 8p23 is known as a region that is associated with loss of heterozygosity (LOH), which is frequently deleted in hepatocellular carcinoma (HCC) tissues. We report here the characterization of a gene for a liver-related putative tumor suppressor (LPTS) localized at 8p23, that was isolated by allelic-loss mapping and positional candidate cloning. The expression of the gene for LPTS was ubiquitous in normal human tissues, albeit at relatively low levels, whereas levels appeared to be significantly reduced, or sometimes undetectable in HCC cells and neoplastic tissues. Thus, it appeared that LPTS might be involved in the control of cell proliferation. Indeed, we observed the significant suppression of growth and growth arrest of SMMC-7721 HCC cells after introduction of the gene for LPTS. We also used antisense oligodeoxynucleotides (AS-ODNs) to suppress the expression of LPTS in normal liver cells L02. Several AS-ODNs specific for LPTS mRNA significantly enhanced cell growth, whereas control oligodeoxynucleotides (ODNs) did not. Our results suggest that LPTS might be a growth-inhibitory protein in human hepatocytes. (HEPATOLOGY 2000;32:721-727.)

Abbreviations: HCC, hepatocellular carcinoma; LOH, loss of heterozygosity; STS, sequence-tagged site; HBV, hepatitis B virus; EST, expressed sequence tag; cDNA, complementary DNA; LPTS, liver-related putative tumor suppressor; Ks, samples of primary HCC; Ls, adjacent samples of nontumorous tissues; Ns, normal liver tissues; DMED, Dulbecco’s Modified Eagle Medium; FCS, fetal calf serum; PCR, polymerase chain reaction; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; RT-PCR, reverse transcription-polymerase chain reaction; S-ODN, sense oligodeoxynucleotide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; AS-ODN, antisense oligodeoxynucleotide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; S-ODN, sense oligodeoxynucleotides; ODN, oligodeoxynucleotide; cM, centi Morgan.

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The nucleotide sequence of the gene for LPTS has been submitted to the EMBL/GenBank sequence database and is available under accession number AF205718.

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Human hepatocellular carcinoma (HCC) is one of the most common malignancies of the liver. Similar to other malignancies, HCC manifested the abnormalities of chromosomes such as deletions, duplications, and translocations, but the molecular mechanism responsible for the malignant phenotype has not yet been elucidated. Comparative genomic hybridization revealed that human chromosome 8p23 is one of the loci associated with alterations such as loss and gain.1,2 It has been shown that sequential accumulation of genetic alterations in the genome plays a central role in the development of cancer, including the activation of protooncogenes and the inactivation of tumor suppressor genes.3,4 Protooncogenes, such as ras, are rarely changed in primary HCC and appear not to be related in any major way to the pathogenesis of HCC.5 By contrast, chromosome deletions at the sites of tumor suppressor genes have been frequently detected in many cases of HCC, suggesting that the inactivation of tumor suppressor genes might be critical for the development of HCC. However, no relevant tumor suppressor gene has yet been identified in patients with HCC.

Somatic inactivation of a tumor suppressor gene, usually achieved by intragenic mutation in one allele of the gene with subsequent loss of a chromosome region that spans the second allele, showed loss of heterozygosity (LOH).6 Mapping of the hotspot regions of tumor suppressor genes can be achieved by examining regions with high-frequency LOH. Among the various deleted chromosome regions identified by several laboratories, chromosome 8p has a particularly high frequency of LOH.1,3,6-9 Approximately 60% of HCC tumors were shown to exhibit LOH at 1 or more loci at 8p with 3 distinct, minimal deleted regions, namely 8p21 (13 centi Morgan [cM]), 8p22 (9 cM), and 8p23 (5 cM).10 The microsatellite marker D8S277, located at 8p23, is a possible sequence-tagged site (STS) landmark for such LOH in cases of HCC.6

Chromosome 8p has also been shown to be a site of integration of hepatitis B virus (HBV) in HBV-positive cases of HCC.11 The integration of HBV appears to be a major risk factor in hepatocarcinogenesis, contributing to the development of HCC by disrupting the chromosomal region that includes a tumor suppressor gene(s). Histological analysis has shown that loss of chromosome 8p23 plays a key role in the progression of HCC.12,13

We used the strategy known as positional candidate cloning to select the marker D8S277 at 8p23. We identified several expressed sequence tags (ESTs) close to D8S277. Using these ESTs, we isolated the cDNA for a novel liver-related
putative tumor suppressor gene (LPTS) from a cDNA library prepared from human hepatocytes. We then examined the effects of expression of LPTS on the proliferation of normal liver cells (L02) and HCC cells (SMMC-7721). Our results suggest that LPTS, encoded by a gene on chromosome 8p23, might be a growth-inhibitory protein in liver cells.

MATERIALS AND METHODS

Samples of Primary HCC, Adjacent Tissues, and Cell Lines. All samples of primary HCC (Ks), adjacent samples of nontumorous tissues (Ls), and samples of normal liver tissues (Ns) were obtained from Eastern Hepatobiliary Surgery Hospital (Shanghai, P.R. China). The serial numbers of samples were those recorded by the hospital. All tested patients with HCC, with the exception of K20 and K16, were positive for the HBV surface antigen. The samples of normal tissue were from patients with cavernous hemangioma and were negative for the HBV surface antigen. All tissues were placed in liquid nitrogen immediately after surgical resection. The HCC cell lines HepG2, SMMC-7721, and Bel-7402 and the line of normal liver cells L02 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, P.R. China). HepG2 and Bel-7402 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies Inc., Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Life Technologies Inc.) and L02 and SMMC-7721 cells were cultured in RPMI medium 1640 (Life Technologies Inc.) and L02 and SMMC-7721 cells were cultured in RPMI medium 1640 (Life Technologies Inc.) plus 10% FCS.

Cloning of cDNA for LPTS. We designed primers P1 (5'-CTGACGCCGAAGTAGACT-3') and P2 (5'-CTGACAGTAATGCACGCATGTTTCGTTCCCTGATT-3') by reference to the sequences of EST AA700052 and H22495, which shared with that of unigene Hs.99829. We introduced a primer, P1 (5'-CAAGATGAGATGAGCAGG-3') and P2 (5'-CTGACAGATGTACGGCACTGTTTGTGATGATG-3'), into the MTT assay (see later) after 2, 4, and 8 days.

Quantitation of Viable Cells. The MTT colorimetric assay was performed to determine the ability of viable cells to convert a soluble tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a formazan precipitate, corresponding to the number of viable cells. Ten microliters of MTT (5 mg/mL in PBS) solutions were added to each well of the 96-well microtiter plates (Corning Inc., Corning, NY) at approximately 1.5 x 10^5 cells in 100 μL of medium per well. After cells had adhered to the plate overnight, the medium was replaced with fresh medium that contained 5 μmol/L AS-ODNs. The medium was replaced with fresh medium plus AS-ODNs at the same concentration for 1-2 days. Viable cells were counted by the MTT assay (see later) after 2, 4, and 8 days.

RESULTS

We selected candidate ESTs for positional cloning by reference to Genemap98 from NCBI (http://www.ncbi.nlm.nih.gov/genemap). Ten ESTs around the marker D8S277 were selected for further analysis. We then identified EST AA700052, which was derived from a cDNA library prepared from human liver cells. EST AA700052 belonged to the unigene cluster Hs.99829. Using available information about Hs.99829, we recovered a 1.3-kb cDNA fragment after RT-PCR with EST primers from normal liver tissue and then cloned the fragment into the T-easy vector (Promega Co., Madison WI), and the nucleotide sequence was determined with a fluorescent automated DNA sequencer (TaKaRa Inc., Kyoto, Japan).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was reverse transcribed in a 20-μL reaction mixture that contained 1 μg of total RNA from normal liver, cancerous liver or adjacently noncancerous liver, or from cultured cell lines, 2.5 pmol of oligo dT-adaptor primer and 1 μL of AMV reverse transcriptase XL (5 units, TaKaRa Inc.). The reaction mixture was incubated at 42°C for 30 minutes and then boiled to inactivate the enzyme. The reaction mixture was then divided into 2 parts for detection of the target gene and for PCR with β-actin mRNA as the internal control. The primers for detection of the level of the RNA were designated LPTS (P1 and P3, 5'-CTGACGCCCAAAAAGAGGAGAGG-3') and β-actin (β-actin1, 5'-TGACGGGTCACCCACACGTGTCGGCTCATT-3'; β-actin2, 5'-CTGACAGTTGCGTGCAGATGAG-3'), respectively. PCR was performed as follows: 94°C for 2 minutes and then 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds. The products of PCR were separated by electrophoresis on a 1% agarose gel.

Formation of Stable Colonies of Transformed Cells. HCC cells (SMMC-7721, approximately 2 x 10^6 cells per 60-mm dish) were transfected with 2 μg of pcDNA3 (Invitrogen, Inc. Carlsbad CA) that included the full length cDNA for LPTS or pcDNA3, the control vector, with LipofectAMIN reagent (Life Technologies, Inc.) according to the manufacturer's protocol. Then, 24 hours after transfection, G418 (Life Technologies, Inc.) was added to the medium at a final concentration of 700 μg/mL. Selection was continued for 3 weeks. Colonies were fixed with methanol and stained with Giemsa for enumeration of transformed cells. Transformed colonies were confirmed by amplification of the neo gene with the primers neo1 (5'-CAAGATGAGATGAGCAGG-3') and neo2 (5'-CCGGCTAGAGAATCTTCGCT-3').
and SwissProt databases revealed 3 extensively homologous sequences in Caenorhabditis elegans (PID: e214883), Drosophila melanogaster (PID: g7302356), and Saccharomyces cerevisiae (PID: g6594229). The deduced LPTS protein was 45% homologous to a 339 amino acid coding region of C. elegans, 46% homologous to a 726 amino acid coding region of D. melanogaster, and 33% identical to a 271 amino acid coding region of S. cerevisiae (Fig. 2). In the 3’-end untranslated region, we identified polyadenylation signals AATAAA at positions 701 and 1261. The cDNA appeared to represent the expressed gene around the D8S277 landmark at the LOH locus.

We analyzed HCC, normal liver tissue, and HCC cell lines by RT-PCR in an effort to detect the expression of the gene for LPTS using primers that corresponded to the 3’-end of the untranslated region of the gene. As shown in Fig. 3A and B, we detected variable levels of expression of the gene in HCC samples. An amplified fragment of 328 bp DNA was detected region, we identified polyadenylation signals AATAAA at positions 701 and 1261. The cDNA appeared to represent the expressed gene around the D8S277 landmark at the LOH locus.

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in the analysis of 6 samples of normal liver tissue (N1, N3, N4, N5, N14, and N15), in a cDNA library prepared from normal liver (Life Technologies, Inc.) and in 1 normal hepatocyte (L02). Among 8 samples of HCC, K26, K53, K54, and K57 yielded a band that corresponded to the amplified 328-bp DNA, whereas no similar band of amplified DNA was generated from the samples of K14, K16, K20, and K59. We also examined the expression of the gene for LPTS in 4 pairs of HCC samples and the corresponding adjacent noncancerous tissue by Northern blotting (Fig. 3C). The results clearly showed the lower level of expression of the gene for LPTS in three (K91, K96, and K11) of the 4 samples of HCC, as compared with the respective adjacent tissues. We also examined the expression of LPTS gene in 3 lines of HCC cells, namely SMMC-7721, Bel-7402, and HepG2. The gene for LPTS was expressed only in HepG2 cells (Fig. 3A). Taken together, these results indicate that LPTS is generally not expressed in HCC cells.

We next examined whether LPTS could repress the proliferation of the HCC cell line SMMC-7721, which is a well-characterized line of HCC cells but was found not to express endogenous LPTS, by introducing the gene encoded for LPTS. We used colony formation assay with selective pressure from G418 for 3 weeks and found a consistent reduction of approximately 70% in colony number when cells had been transfected with the LPTS-expression vector as compared with the control vector (Fig. 6A and B). This effect was reproducible and was observed in more than 3 independent transfection experiments. We used PCR to amplify the neo gene from these colonies and showed that they were indeed neomycin resistant colonies (Fig. 6C). Thus, suppression of clonal outgrowth was clearly the result of the transfection of the LPTS-expression vector. We pooled colonies and examined the expression of the LPTS transcript by Northern blotting. The level of the LPTS transcript was still very low, but a little higher than that in cells transfected with the control pcDNA3 (data not shown). These results indicated that the gene for LPTS could suppress the proliferation of HCC cells.

**DISCUSSION**

We successfully cloned the cDNA for a novel human HCC-related gene, the gene for LPTS, by positional candidate cloning using mRNAs derived from normal human liver tissue as template and mapping information in GenBank Genmap98. The cDNA for LPTS included a complete open reading frame from nt 133 to 657 and encoded a protein of 174 amino acids.
The length of the cDNA fragment was consistent with the
length of the mRNA for LPTS.

The gene for LPTS mapped to chromosome 8p23, a locus
with high-frequency LOH (about 42%), near the microsatel-
lite marker D8S277. Molecular analysis of HCC has suggested
that there is more than 1 tumor suppressor gene on chromo-
some 8p23. The gene for LPTS, located at 8p23, was deleted in
more than 50% of patients with HCC that we analyzed, and
such a frequency is similar to the frequency of LOH (42%). A
growing body of evidence suggests that chromosome 8p is
active in many types of carcinogenesis and metastasis and that
more than 1 tumor suppressor gene is located at 8p. Studies of
LOH and comparative genomic hybridization have revealed
that these genes are inactivated not only in HCC, but also in a

![FIG. 5. Effects of antisense oligodeoxynucleotides (AS-ODNs) tar-
goted against LPTS mRNA on the prolifera-
tion of human L02 hepatocytes. (A) Schematic repre-
sentation of the positions of AS-ODNs targeted
to LPTS mRNA. (B) Nucleotide sequences of the AS-ODNs that were
specifically targeted to LPTS mRNA and the controls. All AS-ODNs (AS1
through AS5), S-ODNs (S2 and S3),
and the random ODN were syn-
thetized by the phosphorothioate
method. (C) Effects of AS-ODNs specific for LPTS mRNA on the
proliferation of L02 cells. The MTT as-
say was used to quantitate the cell
growth in the presence and absence
of AS-ODNs, S-ODNs, and the ran-
dom ODN. The graphs show the av-
erages of results of 3 experiments
with standard deviations. In the plot,
the cell growth with AS-ODNs,
S-ODNs, and random ODN added in
is indicated by ▲ and cell growth
without AS-ODNs, S-ODNs, or ran-
dom ODN added in uses the symbol
of ■. The results were the means
from five independent experiments
with standard deviations (SD).

![FIG. 6. Suppression of clonal outgrowth by LPTS in hepatocellular
carcinoma cell line SMMC-7721. (A) Expression of LPTS suppressed the
colony formation in SMMC-7721 cells. SMMC-7721 cells were trans-
fected with either the pcDNA3 vec-
tor as a control (left) or with the
LPTS-expression vector (right).
Transfected cells were then selected
by incubation with G418 for 3
weeks. Cells were fixed, stained with
Giemsa, and photographed. (B) Per-
centage of G418-resistant (NeoR)
colonies that formed relative to the
number formed after transfection
with pcDNA3 (defined as 100% in
each experiment). Data are results
from 3 independent transfections
with SD. (C) Expression of neo gene
of each G418 resistant colony. PCR
was performed to amplify the neo
gene from colonies that had been
picked randomly from the dishes.
The 760-bp fragment of amplified DNA that carrying neo is indicated
by an arrow. M, molecular marker; 1,
SMMC-7721 cells as a negative con-
trol; 2 through 5, 4 colonies picked
randomly as sources of template DNA.
The frequencies of deletion of chromosome 8p are sometimes even higher than those of chromosomes 17p and 13q, which harbor the tumor suppressor genes p53 and Rb, respectively. It was reported that microcell-mediated transfer of chromosome 8 suppressed tumorogenesis or the proliferation of colorectal, breast, and colon cancer cells. Thus, chromosome 8p seems likely to include several tumor suppressor genes with different roles in human malignancies. To date, however, there have been no reports of the identification of a tumor suppressor gene on 8p. The gene for LPTS appears to be a strong candidate for a tumor suppressor gene. It is located in a high-frequency LOH region of chromosome 8p23. In normal human tissues, we detected the expression of the LPTS transcript at different levels. By contrast, more than 50% of samples of HCC did not express the gene for LPTS, whereas samples of normal liver adjacent to HCC did generate evidence for expression of the gene (Fig. 3). Thus, expression of the gene for LPTS was detected in normal tissues (Fig. 4), but was frequently undetectable in HCC. Such patterns of expression are typical of tumor suppressor genes. We are now performing the experiments to identify the mutation sites of the gene for LPTS.

To explore the possible roles of LPTS in the control of cell proliferation, we used AS-ODNs in an attempt to suppress its expression. The proliferation of L02 hepatocytes by AS-ODNs specific for the LPTS transcript was significantly enhanced as compared with that of control L02 cells (Fig. 5C). To determine whether ectopic expression of LPTS could modulate the proliferation of HCC cells, we introduced LPTS cDNA into SMMC-7721 cells. As a result, we detected a significant reduction in the proliferation of HCC cells. The stimulatory effects of specific AS-ODNs on L02 cells and the suppressive effect of the LPTS cDNA on HCC cells showed that the gene for LPTS is a growth-arrest gene that acts directly or indirectly to control the proliferation of cells. The expression of tumor suppressor genes is usually down-regulated in cancerous tissue and significant in the normal tissues. The products of such genes regulate cell growth and differentiation in a negative fashion and, thus, suppress neoplastic development. It is possible that the total inactivation of the gene for LPTS as a result of deletions or mutations might result in uncontrollable cell growth, possibly leading to the development of a tumor. The tumor-suppressive function of the gene for LPTS and its role in apoptosis require further investigation.

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