DLC-1 Is Deleted in Primary Hepatocellular Carcinoma and Exerts Inhibitory Effects on the Proliferation of Hepatoma Cell Lines with Deleted DLC-1

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Abstract

We investigated the expression and deletion of DLC-1 (frequently deleted in liver cancer gene), first reported in 1998 and having a high homology with rat p122RhoGAP in hepatocellular carcinoma (HCC). Six (20%) of 30 human HCC samples and 2 (40%) of 5 HCC cell lines were found to have no detectable DLC-1 expression by reverse transcription-PCR. Homozygous DLC-1 deletion was detected by Southern blotting in two of six HCC samples and in both HCC cell lines with no DLC-1 expression. Transfection of DLC-1 into 5 HCC cell lines (two with DLC-1 deletion and three with intact DLC-1) showed significant growth inhibition in these two HCC cell lines with deleted DLC-1 with both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and colony formation assays but not in three other HCC cell lines with intact DLC-1. Our findings suggest that DLC-1 may play an important role in hepatocarcinogenesis.

Introduction

HCC is one of the most common cancers worldwide. The identification of genes associated with human HCC is critical in understanding the molecular mechanisms of carcinogenesis and important for the diagnosis of HCC. A new gene frequently deleted in liver cancer (the DLC-1 gene), located at chromosome 8p21.3–22, was first reported by Yuan et al. (1) in 1998. The full-length cDNA of DLC-1 is of 3850 bp that encode a protein of 1091 amino acid residues. The DLC-1 sequence has an 80% homology with rat p122RhoGAP (1), a GTPase-activating protein for Rho family proteins. In human and other mammalian cells, RhoGAPs are important regulators in the switching between the active GTP-bound state and the inactive GDP-bound state of Rho proteins. They stimulate the intrinsic GTPase activity of Rho proteins and catalyze the conversion of the active GTP-bound Rho proteins to the inactive GDP-bound ones. This attenuates the signal-transducing activities of Rho proteins, and RhoGAPs thus act as a negative modulator. The Rho family proteins function as important regulators in the organization of actin cytoskeleton. Small GTPases of the Rho family have been shown to be regulators in the organization of the actin stress fibers related to microfilamental network and cell-cell contact. These Rho proteins are also involved in Ras-mediated oncogenic transformation. In tumors, the Rho-small GTPases have recently been reported to be overexpressed, suggesting that Rho-GTPases are involved in human carcinogenesis (6). DLC-1 sequence has a high homology to RhoGAP (7). However, the role of DLC-1 in the growth of human HCC is not clear. To investigate the status of DLC-1 in human HCC, we evaluated its expression in 30 cases of human HCC samples and in 5 HCC cell lines by RT-PCR. Homozygous deletions were examined by Southern blot analysis. We also investigated the effects of DLC-1 on HCC cell proliferation.

Materials and Methods

Tumor Samples and Cell Lines. Paired samples of HCC and corresponding nontumorous liver tissues from 30 Chinese patients who had had surgical resection of the tumors were used in this study. A liver specimen from a patient with liver metastasis from a primary colorectal cancer was used as normal liver tissue. All specimens were obtained immediately after resection, snap-frozen in liquid nitrogen, and kept at −70°C. The five human HCC cell lines used were: Huh-7 (a gift from H. Nakabayashi, Hokkaido University School of Medicine; Ref. 8); SMMC-7721 (9) and BEL7402 (Ref. 10; both gifts from Shanghai Institute of Cell Biology, Chinese Academy of Sciences); and HepG2 and Hep3B (both obtained from American Type Culture Collection). They were maintained in DMEM medium supplemented with 10% fetal bovine serum and 100 units/ml of penicillin and streptomycin.

Plasmids and Oligoprimers. According to the published sequence of DLC-1 (1), and using 195-366 bp from the template that contains the full-length coding sequence of the gene, a pair of primers (primer set 1) was designed for PCR. Primer set 1 sense: 5’-TGGTCAAGAGAGCATGAT-3’; antisense: 5’-CTTCAAGGACTGGCAAAACCTC-3’. At the same time, a 540-bp DNA fragment of DLC-1 was amplified using primer set 1 with sense: 5’-TGGTCAAGAGAGGAGCATGAT-3’; antisense: 5’-TGAAGCTTGAAGGCTGACAGT-3’. Primer set 3: (β-actin) sense, 5’-GTGGGCGCGCCCAGGCAACA-3’; and antisense, 5’-CTCCCTAAATGTCACGCCACGATTTC-3’.

For constructing the plasmid pUC18/DLC-1, the 3.5-kb DLC-1 PCR product was amplified with set 1 primers from normal liver tissue was cloned into pUC18 vector clone site at the Smal site with the Sureclone Ligation Kit (Pharmacia Biotech, Peapack, NJ). The orientation of the DLC-1-inserted fragment was determined by EcoRI digestion. For constructing the expression plasmid pcDNA3.1(−)/DLC-1, a fragment with the entire DLC-1 coding sequence was isolated from pUC18/DLC-1 after BamHI and KpnI digestion and, after confirmation by sequencing, subcloned into BamHI and KpnI sites of pcDNA3.1(−). The recombinant plasmid was transformed into DH5α Escherichia coli, and plasmid DNA was isolated with the Miniprep Kit (Bio-Rad Laboratories, Hercules, CA). pcDNA3.1(−)/wild-type p53 was a gift from Dr. B. Vogelstein of The Johns Hopkins Medical School.

RNA Extraction and RT-PCR. Total RNA was extracted from fresh-frozen human HCC and their corresponding nontumorous liver tissues from the same patients with TRIZOL reagent, as described by the manufacturer (Life Technologies, Inc., Grand Island, NY). cDNA was synthesized from 2 μg of total RNA by using oligo(dT)18 as the primer and with the Expand Reverse Transcriptase Kit (Boehringer Mannheim, Mannheim, Germany). PCR amplification using set 1 primers was performed with the Expand Long Template PCR system (Boehringer Mannheim). A pair of primers (primer set 2) was used to amplify the 461–967-bp region of DLC-1. At the same time, a 540-bp fragment of β-actin was amplified by using set 3 primers as a control.

Southern Blot Analysis. Genomic DNA from the HCC samples, their corresponding nontumorous livers, and HCC cell lines was digested overnight.
with BamH I, EcoRI, and Hind III (all from Amersham, Buckinghamshire, United Kingdom) before Southern blot analysis with the 506-bp DLC-1 (from nucleotides 461 to 967) and β-actin probes.

**Cell Line Transfection.** Cells were seeded at 6 × 10^5 cells in a 25-cm² flask 1 day before transfection. The cells were transfected with 4 μg plasmid DNA using 12 μl FuGENE 6 as described by the manufacturer (Boehringer Mannheim). After 48 h, the medium was replaced by fresh DMEM with Geneticin (G418) to select the transfected cells.

**MITT Assay.** The transfected cells were plated in 96-well microtiter plates at a density of 2 × 10^3 cells/well. They were further cultured for 24, 48, 72, and 96 h, after which the medium was replaced with 100 μl of fresh serum-free medium containing 50 μg MTT. Three h later, the color reaction was quantified with an ELISA plate reader at a test wavelength of 570 nm and a reference wavelength of 630 nm. The cell proliferation rates for the cell lines transfected with pcDNA3.1(−) alone and with pcDNA3.1(−)/DLC-1 were compared. The entire experiment was performed four times independently.

**Colony Formation Assay.** The cells were plated in six-well plates at a density of 1 × 10^5 cells/well. Twelve h later, they were transfected with 2.0 μg of plasmid/well. The cells were then seeded onto six-well plates at a density of 3 × 10^4 cells/well 48 h later, with G418 added at 0.7 mg/ml. The growth of the colonies was examined 2 weeks later with Giemsa stain.

### Results

**Detection of DLC-1 Expression in HCC.** To examine the expression of DLC-1 in human HCC samples and cell lines, RT-PCR was performed. To validate the PCR results, the same cDNA was amplified for β-actin and the results indicated β-actin could be amplified in all samples at comparable levels. Six (20%) of 30 HCC cases had no detectable expression of DLC-1 (Fig. 1A). In addition, DLC-1 mRNA was not detected in two (Huh-7 and BEL7402; 40%) of the 5 HCC cell lines (Fig. 1A).

**Southern Blotting.** To further examine if the absence of expression of DLC-1 was related to genomic alteration of the gene, Southern blot was performed. Southern blot analysis of the genomic DNA of normal liver tissue revealed a fragment of approximately 2.5 kb (BamHI), 2.7 kb (EcoRI), and 3.4 kb (HindIII), respectively, and hybridizing with the DLC-1 probe. Homozygous deletion of DLC-1 was detected in two of six tumor samples and in two HCC cell lines (Huh-7 and BEL7402) in which DLC-1 was not expressed (Fig. 1B). The blot was hybridized with the β-actin probe and the results revealed the presence of a band of expected size in all samples. Therefore, significant numbers of the HCC and HCC cell lines had DLC-1 deletion.

**Effects of DLC-1 on Cell Proliferation in HCC Cell Lines.** To examine the effect of DLC-1 on the growth of HCC cell lines, the full-length DLC-1 gene was cloned from cDNA isolated from a normal human liver sample. A 3474-bp fragment of the DLC-1 gene was successfully amplified and cloned into pUC18 clone vector. The results of sequence analysis demonstrated that the sequence of cloned DLC-1 is identical to the sequence published in GenBank (accession no. AF035119) except at three positions (1148: G→A; 2609: T→A; 2638: T→A). The changes of nucleotides represent changes of serine to asparagine at codon 275, valine to glutamine at codon 762, and cysteine to serine at codon 772, respectively. The results were confirmed by repeated PCR and sequencing, and the changes of the nucleotides are likely to be attributable to the polymorphism of the gene.

To examine the effects of DLC-1 on the cell proliferation in HCC cell lines after transfection, MITT assay was carried out. After the cells were transfected with pcDNA3.1(−)/DLC-1, significant growth inhibition was observed in Huh-7 and BEL7402 cell lines (both with DLC-1 deletion) at 24 h and persisted through 48 and 72 h to 96 h (\( P < 0.001 \) at all time points; Fig. 2A). No significant growth inhibition was seen in the three other HCC cell lines (SMMC, Hep3B, and HepG2), which had normal DLC-1 expression. To rule out whether the effect of DLC-1 on cell proliferation was attributable to different expression levels of the gene, the mRNA levels of DLC-1 in these cell lines were examined. The data indicated that DLC-1 mRNA levels were similar among the transfected cell lines (Fig. 2B).

To examine the more long-term cell proliferation, colony formation assay was performed in the BEL7402 HCC cell line with deleted DLC-1. Significant reduction of colony formation was seen in the HCC cells transfected with pcDNA3.1(−)/DLC-1 or pcDNA3.1(−)/wild-type p53 (Fig. 3). A significant reduction in both colony number and size was observed in the DLC-1-transduced HCC cell line with deletion of DLC-1. p53 was previously shown to inhibit HCC growth (11). The data suggest that DLC-1 exhibited a similar level of growth inhibition.

### Discussion

In this study, the expression of the DLC-1 gene was investigated in 30 cases of human HCC and 5 HCC cell lines. DLC-1 mRNA was undetectable by RT-PCR in 20% and 40% of the human HCC and HCC cell lines (Huh-7 and BEL7402), respectively. In addition, homozzygous deletion was detected by Southern blot analysis in two of six HCCs and in both HCC cell lines in which DLC-1 was not expressed. In the remaining four cases without DLC-1 deletion but with loss of mRNA expression, epigenetic silencing by mechanisms such as aberrant methylation of the DLC-1 promoter might have been responsible. Genetic alterations of the gene cannot be excluded, however, although mutations of DLC-1 in other cancers are low (12). Overall, these results show that the deletion and loss of mRNA expression of DLC-1 are frequent in HCC and HCC cell lines. These findings are in accordance with those reported by Yuan et al. (1), as they found loss of heterozygosity of DLC-1 in about 50% of human HCC, and DLC-1 deletion in 28% of liver cancer cell lines. Both studies suggest that DLC-1 may be a candidate tumor suppressor gene and may play an important role in hepatocarcinogenesis.
In the present study, the effect of wild-type DLC-1 expression in HCC was investigated. Transduction of DLC-1 into two HCC cell lines with DLC-1 deletion resulted in significant inhibition of cell growth by both MTT and colony formation assays. However, no growth inhibition was seen in three other HCC cell lines with intact DLC-1. This difference in growth inhibition was not caused by variation in expression of transfected DLC-1, as the DLC-1 mRNA levels in these cell lines were similar, as shown by semiquantitative RT-PCR. This demonstrates that DLC-1 exerts inhibitory effects on the cell proliferation of HCC cells that are deficient in DLC-1.

DLC-1 was found to be located at chromosome 8p21.3–22 (1). Chromosome 8 has been shown to have frequent deletions and amplifications in solid tumors. Allelic losses from 8p have been documented in cancers including prostate, ovary, colorectal, breast, and lung carcinomas detected by microsatellite analysis (13–15). In HCC, loss of chromosome 8p is one of the most frequent genetic alterations detected by comparative genomic hybridization (16). Furthermore, as many as three separate regions on 8p in HCC are hypothesized to contain a tumor suppressor gene(s) (17). High-density allelotyping showed that at least three regions on 8p (8p21, 8p22, and 8p23 of 13, 9, and 5 cM regions, respectively) might harbor tumor suppressor genes (17). Moreover, a recent study, using comparative genomic hybridization to analyze the differences in genomic alterations between primary HCC tumors and their matched metastatic lesions, shows that significant losses of 8p were detected in 8 of 10 metastatic tumors but in only three corresponding primary tumors (18). The result further suggests that the deletion of chromosome 8p may inactivate a tumor suppressor gene related to HCC metastasis. The location of DLC-1 at chromosome 8p21.3–22 coincides with the frequently deleted region on 8p in HCC. The findings of frequent, undetectable expression and deletion of DLC-1 in HCC and its growth inhibitory effect on HCC cell lines in the present study highlight its importance. These results suggest that DLC-1 may play an important role in hepatocarcinogenesis.

The DLC-1 gene has an 80% homology with rat p122RhoGAP, suggesting that the encoded protein is a human homologue of rat RhoGAP protein. There is strong evidence demonstrating that RhoGAPs play important roles in signal transduction pathways regulated by GTP-binding proteins. At least three subfamilies of the Rho family are substrates for RhoGAPs, namely Rho, Rac, and G25K/cdc42, which are small GTPases (19). Rac and Rho are key elements in the signal-transduction pathways. Rho activates at least two separate events, stress fiber formation and transformation by oncogenic Ras (20). RhoA is thought to interfere mainly with stress-fiber formation (3), and Rac and cdc42 are believed to regulate the formation of lamellipodia and filopodia, respectively (5). The Rac and cdc42 are also integral parts of a biochemical route linking cell surface receptors to c-Jun-NH2-terminal kinase (JNK), the kinase responsible for phos-
phorylating the transactivating domain of the c-Jun protein in vivo (4). Consistent with the notion that RhoA and Rac are essential factors for Ras-mediated transformation (20), a recent study shows that the RhoGAP domain of p190, a Tyr-phosphorylatable G protein, suppresses Ras-mediated tumorigenesis by attenuating Rho/Rac/cdc42 signaling (21). Furthermore, the levels of small Rho-GTPases are presses Ras-mediated tumorigenesis by attenuating Rho/Rac/cdc42 signaling (21). Furthermore, the levels of small Rho-GTPases are involved in a number of tumors, suggesting that Rho-GTPases may be involved in human tumorigenesis (4).

To conclude, the frequent nonexpression and deletion of DLC-1 in HCC, and its growth inhibitory effect in HCC cell lines, show that the DLC-1 may play an important role in hepatocarcinogenesis.

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References


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