Genetic and Epigenetic Alterations of DLC-1 Gene in Hepatocellular Carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common fatal cancers in the world. However, the underlying molecular mechanisms contributing to hepatocarcinogenesis are still unclear. A putative tumor suppressor gene, namely DLC-1 (frequently deleted in liver cancer) was identified and mapped at chromosome 8p21.3–22, a recurrently deleted region in human cancers. The gene exerts inhibitory effects on the cell proliferation of HCC cells. In this study, we investigated the biological function, and genetic and epigenetic status of this gene in human HCC. With in vitro GTPase activating proteins activity assay, we established that DLC-1 protein was a GTPase-activating protein specific for RhoA and Cdc42. Deletion of the DLC-1 gene was frequent in human HCC, as revealed by loss of heterozygosity analysis performed on 100 human HCC cases with markers mapped at the DLC-1 locus, and allelic losses ranging from 44% to 50% of the informative cases. However, somatic mutations of the DLC-1 gene were rare. Moreover, with real-time quantitative PCR, we found that DLC-1 mRNA was significantly underexpressed in HCCs when compared with the corresponding nontumorous livers (P < 0.0001). In addition, the CpG island 5′ to the DLC-1 gene was methylated in 3 of 7 HCC cell lines and in 6 (24%) of 25 primary HCCs. These data suggest that transcriptional silencing by hypermethylation may contribute to the inactivation of the DLC-1 gene. Taken together, the results of our study suggest that both genetic and epigenetic alterations play an important role in inactivation of the DLC-1 gene in hepatocarcinogenesis.

INTRODUCTION

HCC is one of the most common cancers in the world, with the highest incidence in Southeast Asia and sub-Saharan Africa. Epidemiological factors for HCC are well defined, and hepatitis B and C virus infection, cirrhosis, and aflatoxin B1 intake are closely associated with the development of HCC (1). However, the molecular mechanisms leading to the development and progression of HCC remain unclear.

Genome-wide studies using comparative genomic hybridization or LOH1 have shown that chromosomal alterations are frequent in HCC (2–6) as well as in other solid tumors (7–10). In HCC, deletions of chromosomal materials are of a nonrandom pattern, and chromosome arms including 1p, 4q, 8p, 13q, 16q, and 17p have been found to be more susceptible to allelic losses (4, 5, 11). Accumulation of allelic losses is associated with more advanced tumor stages and a more aggressive tumor behavior, suggesting that continuous loss of chromosomal materials during tumor progression may lead to inactivation of genes that regulate cell growth and adhesion, therefore conferring selection advantage for increased aggressiveness of the tumor (11).

A candidate tumor suppressor gene named frequently deleted in liver cancer (DLC-1) was isolated recently from human HCCs by PCR-based subtractive hybridization approach (12). The DLC-1 gene was mapped at 8p21.3–22, a region suspected to harbor tumor suppressor genes and recurrently deleted in HCC as well as other solid tumors (2, 13–15). The DLC-1 sequence shares high homology with rat p122RhoGAP, a GTPase-activating protein for Rho family proteins (16). GTPases of the Rho family are members of the Ras superfamily of small GTP-binding proteins that act as molecular switches to regulate various cellular signaling pathways (17). Thus far, 18 members of the Rho family proteins have been identified. RhoA, Rac1, and Cdc42 are three representative and well-studied members. Rho family proteins play an essential role in regulating diverse biological functions including cytoskeletal organization, cell adhesion, and cell cycle progression (18–21). Recent evidence suggests that hyperactivation of Rho family proteins are implicated in tumorigenesis (22–24). The activity of small GTPase protein depends on the balance between active GTP-bound and inactive GDP-bound states, which are controlled by the intrinsically GTPase activity and their regulatory proteins. Guanine nucleotide exchange factors promote the active GTP-bound state by facilitating the exchange of GDP by GTP. GAPs, on the other hand, are the negative modulator of small GTPase proteins by stimulating the intrinsic GTPase activity and converting them into an inactive GDP-bound state (25, 26). Thus, RhoGAPs serve as tumor suppressors by balancing the oncogenic potential of Rho proteins. Consistent with this notion, the reintroduction of DLC-1 inhibited the proliferation of DLC-1-defective hepatoma cells (27). However, little is known about its function, as well as the genetic and epigenetic factors contributing to the inactivation of the DLC-1 gene in human HCCs. In the present study, the RhoGAP activity of DLC-1 on RhoA, Rac1, and Cdc42 was evaluated. The genetic and epigenetic alterations, including allelic loss, somatic mutation, mRNA expression level, and 5′CpG island methylation status of the DLC-1 gene in human HCCs were also investigated.

MATERIALS AND METHODS

Tumor Samples and Cell Lines. One hundred paired samples of primary HCC and the corresponding nontumorous liver tissues from Chinese patients were collected at the time of surgical resection at Queen Mary Hospital at the University of Hong Kong between 1992 and 2000. All of the specimens were obtained immediately after surgical resection, snap-frozen in liquid nitrogen, and kept at −70°C. Cell lines used in this study were either obtained form American Type Culture Collection (HepG2, Hep3B, PLC/PRF/5, HLE, and WRL) or Shanghai Institute of Cell Biology (Bel-7402 and SMMC-7721; Ref. 28). They were maintained in DMEM supplemented with 10% fetal bovine serum and 100 units/ml of penicillin and streptomycin.

Plasmids. The GST-DLC-1–fusion plasmid was constructed by isolating a fragment of the entire DLC-1 coding sequence from pcDNA3.1(−)/DLC-1 (27) and subcloning into the BamHI and SalI sites of the GST gene fusion vector, pGEX-4T-1 (Promega, Madison, WI). The DNA sequence and reading frame of recombinant plasmid were confirmed by DNA sequencing.

GST Fusion Protein Purification. GST fusion protein was purified as described previously (29). Briefly, GST-DLC-1 recombinant plasmid was transformed into Escherichia coli, and the expression of GST-fusion protein was induced by isopropyl-β-D-thiogalactopyranoside. Transformed cells were then resuspended in a buffer [150 mM NaCl, 16 mM Na2HPO4, and 4 mM NaH2PO4 (pH 7.3)] containing 2 mM phenylmethysulfonyl fluoride, 2 mM DTT, 2 μg/ml antipain, and 2 μg/ml leupeptin. The transformed cells were lysed by sonication, and GST-DLC-1 fusion protein was purified with glutathione agarose beads. The protein was eluted with 10 mM reduced glutathione and

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1The abbreviations used are: LOH, loss of heterozygosity; HCC, hepatocellular carcinoma; GAP, GTPase activating protein; GST, glutathione S-transferase; RT-PCR, reverse transcription-PCR; nt., nucleotides; 5-Aza-dC, 5-aza-2′ deoxycytidine; MSP, methylation-specific PCR.

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dialyzed in low salt buffer [10 mM Tris (pH 7.6), 2 mM MgCl₂, and 0.1 mM DTT] overnight.

**In Vitro GAP Assay.** GAPase activity was assayed, according to a method described previously (30). Briefly, GST fusion proteins of RhoA, Rac1, and Cdc42 (50 ng) were preloaded with [γ³²P]GTP (600 Ci/mmole) in GTP loading buffer [20 mM Tris-HCl (pH 7.6), 0.1 mM DTT, 25 mM NaCl, and 4 mM EDTA] for 10 min at 30°C. The preloaded small GTPase protein was diluted with dilution buffer [20 mM Tris-HCl (pH 7.6), 0.1 mM DTT, 1 mM GTP, and 1 mg/ml BSA], and the GTP hydrolysis was initiated by the addition of GST-DLC-1 or GST. The samples from different time points were then spotted onto filters and washed with 20 ml of cold assay buffer [50 mM Tris-HCl (pH 7.6), 50 mM NaCl, and 5 mM MgCl₂], and the radioactivity was determined by scintillation counting.

**DNA and RNA Extraction.** High-quality genomic DNA was extracted by phenol-chloroform after proteinase K treatment. Total RNA was extracted by Trizol reagent, according to the manufacturer’s instructions (Life Technologies, Inc., Grand Island, NY). cDNA was synthesized from 1 μg total RNA by GeneAmp RNA PCR Kit (Perkin-Elmer, Foster City, CA).

**Allelic Loss Analysis.** Three fluorescent-labeled microsatellite markers were used for LOH analysis: D8S1827 and D8S552 flank the DLC-1 gene, and D8S1754 is mapped to the 446-bp fragment flanking the RhoGAP domain of the DLC-1 gene. Microsatellite markers were amplified from 50–100 ng DNA extracted from human HCCs and their corresponding nontumorous livers. Reaction was initiated by hot start at 95°C for 12 min, 10 cycles of amplification (94°C for 15 s, 55°C for 15 s, and 72°C for 15 s), and 20 cycles of amplification with lower denaturing temperature (89°C for 15 s, 55°C for 15 s, and 72°C for 15 s) followed by a final elongation at 72°C for 30 min. PCR products were then analyzed on a model 377 automatic DNA sequencer (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions, and the results were analyzed with Genotyper software (Applied Biosystems). Cases were defined as LOH when an allele peak signal from tumor DNA was reduced by 50% compared with their corresponding nontumorous livers.

**DNA Sequencing.** A 446-bp fragment flanking the RhoGAP domain of the DLC-1 gene (nt. 2279–2724 bp) was amplified from 60 cases of HCC and their corresponding nontumorous livers using the following primers: 5′-GTT GCC TCA GAG CAT CCA G-3′ (forward) and 5′-GGG TGT TGA GAT GGA AGA GG-3′ (reverse). The condition of PCR reaction was as follows: 95°C for 12 min, and 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by final extension at 72°C for 10 min. PCR products were purified from 1.2% agarose with Concert nucleic acid purified system (Life Technologies, Inc.) and subjected to direct sequencing on 377 automatic DNA sequencer.

**Real-Time Quantitative RT-PCR.** A 70-bp fragment of the DLC-1 gene (nt. 855–924 bp) was amplified by real-time PCR in ABI Prism 7700 (Applied Biosystems). The real-time PCR reaction was carried out in a total volume of 50 μl containing 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 0.5 μM of each forward and reverse primers, and 0.25 μM of TaqMan probe and 4 μl of synthesized cDNA. The reaction was initiated by carryover decontamination at 50°C for 2 min and hot start at 95°C for 10 min, and followed by 40 cycles of two-step PCR reaction: 95°C for 15 s and 60°C for 1 min. The sequences of primers and TaqMan Probe were as follows: 5′-CCC CGG ACT AAC TCC TCC ATG-3′ (forward), 5′-TGC CGA AAG AGT GCT CAT TG-3′ (reverse), and 6-carboxyfluorescein -CGT CTG TCT CTC CAG CAA CTT GGC-TAMRA (TaqMan probe, nt. 878–902 bp). A housekeeping gene, TATA box binding protein (TBP; Applied Biosystems) was used as an endogenous control (31). The calibration standard curve was set up by three serial dilutions of plasmids of known concentration containing DLC-1 or TBP cDNA insert. Measurements were repeated at least twice to ensure the reproducibility of results.

**Northern Blot Analysis.** Total mRNA extracted from cell lines that showed no expression of DLC-1 mRNA in semiquantitative RT-PCR were transferred to Hybond-n + membrane (Amersham). The blot was then hybridized with a random labeled 3.5-kb fragment released from pCDNA3.1/−/DLC-1, which contains the entire DLC-1 coding sequence. The same blot was extensively stripped and hybridized with a β-actin cDNA probe for RNA loading control.

**Methylation-Sensitive Restriction Enzyme Analysis.** High-quality genomic DNA (0.5 μg) was digested overnight with methylation-sensitive restriction enzyme, either HpaII (CCGG) or Smal (CCCGGG), and purified by ethanol precipitation. A 782-bp fragment flanking the CpG island of the DLC-1 gene (nt. from −445 to +337 bp) was amplified by PCR with primers 5′-GCT ACC AAG AAA AAG AGG GG-3′ (forward) and 5′-GGC TTC TG CAC ATC AA-3′ (reverse). The reaction was carried out in a 25-μl mixture containing 1× PCR buffer, 1× GC-RICH solution (Roche, Indianapolis, IN), 0.25 mM MgCl₂, 1 mM of dNTP, and 0.5 μM of each forward and reverse primers, and 0.5 unit of AmpliTaq Gold (Applied Biosystems) at the following conditions: 95°C for 12 min, 40 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s, and final extension at 72°C for 10 min.

**Sodium Bisulfite Treatment.** Sodium bisulfite treatment was carried out using a protocol modified from Clark et al. (32). Two μg genomic DNA was denatured with 0.3 M NaOH and treated with 3.6 M sodium bisulfite (pH 5.0) at 55°C for 16–20 h. Bisulfite-treated DNA was purified with Wizard DNA cleanup system (Promega). Purified DNA samples were desulfonated with 0.3 M NaOH at room temperature, neutralized with ammonia acetate, ethanol precipitated, and resuspended in 30 μl Tris-EDTA buffer.

**Bisulfite PCR and Sequencing.** Five μl of bisulfite-treated DNA were subjected to PCR using the following primers (nt. 45–336 bp): 5′-GTG TTT AGT TAG GAT ATG GT-3′ (forward) and 5′-ACT TCT TTC TAC ACA TCA AAC AC-3′ (reverse). The reaction was carried out at the following conditions: 95°C for 12 min, 45 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and final extension at 72°C for 10 min. Amplified PCR product was purified from agarose gel and cloned into pGEM-T Easy vector (Promega). Bisulfite sequencing was performed on at least 4 individual clones using the 377 automatic DNA sequencer (Applied Biosystems).

**MSP.** Bisulfite-treated DNA was amplified by PCR with methylation status-specific primer pairs, which were able to discriminate between methylated and unmethylated alleles of the DLC-1 gene (33). The primer sequences for the methylation specific PCR were as follows (nt. from −31 to +147 bp): 5′-TGG AAA GAT CGA AAC GAG GGA GCG-3′ (forward) and 5′-CCC AAC GAA AAA ACC GTA ACA ACG-3′ (reverse). The sequences of unmethylation-specific primers were as follows (nt. from −28 to +144 bp): 5′-TTT AAA GAT CAG GAT GAG GGT-3′ (forward) and 5′-AAA CCC AAC AAA AAA ACC ACA CTA ACA-3′ (reverse). Reaction for unmethylation-specific PCR was carried out at the following conditions: hot start at 95°C for 12 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and final extension at 72°C for 10 min. The reaction condition for the MSP was as same as that of unmethylated one, except that the annealing temperature was raised to 58°C.

**5-Aza-dC Treatment of HCC Cells.** Two × 10⁵ cells were seeded into six-well plates and cultured for 24 h, followed by 5-Aza-dC (Sigma) treatment at various concentrations (0.1, 1, 10, 50, and 100 μM) for 4 days. Total RNA was extracted from the cells, and cDNA was synthesized as described above. The expression of DLC-1 mRNA was detected by RT-PCR with specific primers (nt. 192–649 bp): 5′-AGC AAG GAT GCG TTG AGG-3′ (forward) and 5′-CAG CTC TTG CGT CTC TGG-3′ (reverse). A fragment of β-actin was amplified as control. The PCR was terminated at the exponential phases: 30 cycles for DLC-1 and 22 cycles for β-actin, including 1 cycle of hot start at 95°C for 12 min, followed by amplification at 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, and a final extension at 72°C for 10 min.

**RESULTS**

**DLC-1 Is a GAP Protein Specific for RhoA and Cdc42.** To investigate the structure and functional features of DLC-1 protein, the amino acid sequence of DLC-1 was subjected to a database search. Similar to its rat homologue, DLC-1 protein contains a highly conserved amino acid sequence, namely, RhoGAP domain. This RhoGAP domain is identified in many other proteins exhibiting GAP activity toward Rho family proteins (34). Thus, the presence of RhoGAP domain predicts that DLC-1 could be a GAP for Rho family GTPases. To test this possibility, we expressed and purified DLC-1 protein from E. coli and tested the in vitro GAP activity of DLC-1 protein toward three of the most well-characterized members of Rho family proteins, namely, RhoA, Rac1, and Cdc42. DLC-1 significantly stimulated the intrinsic GTPase activity of RhoA and Cdc42. However, DLC-1 did not...
not affect the intrinsic GTPase activity of Rac1 (Fig. 1). These findings suggest that DLC-1 is a GAP protein specific for RhoA and Cdc42.

Frequent Allelic Loss of DLC-1 Locus in Primary HCCs. Our previous study using Southern blot analysis demonstrated that deletion of the DLC-1 gene was not uncommon in hepatoma cell lines and primary HCCs (27). This study continues our investigation into allelic losses of the DLC-1 locus in human HCC. We used three microsatellite markers for LOH analysis, D8S552 and D8S1827, which flank the 5’ and 3’ of the DLC-1 locus, and D8S1754, which is an intragenic marker mapped to the first intron of the DLC-1 gene. In the 100 pairs of HCC cases examined, LOH with these microsatellite markers was found to be 44.3%, 44.4%, and 50.0% of the informative cases, respectively (Table 1; Fig. 2). These findings showed that allelic loss on the DLC-1 locus was frequent in primary HCCs.

Rare Somatic Mutation of the DLC-1 Gene in Primary HCCs. As DLC-1 is a RhoGAP, we therefore amplified a fragment of the DLC-1 gene encoding the RhoGAP domain by RT-PCR and screened for somatic mutations by direct DNA sequencing. A total of 60 primary HCCs and their corresponding nontumorous livers were examined. However, no somatic mutations could be detected in any of these HCCs, apart from a silent mutation of 2529 C>A (Arg733Arg) that did not result in amino acid substitution in one case. In addition, three polymorphisms, 2325 A→G, 2580 T→A, and 2609T→A were found in all of the HCCs as well as all of the corresponding nontumorous livers when compared with the GenBank data (NM_006094). This may be a population-specific polymorphism or a sequencing error in the GenBank entry.

Reduced DLC-1 mRNA Expression in Primary HCCs. To quantify the mRNA expression level of DLC-1 in human HCCs, real-time quantitative PCR was carried out in 40 pairs of HCCs and their corresponding nontumorous livers. The DLC-1 mRNA level was normalized against the housekeeping gene TBP. Of the 40 cases, 27 (67.5%) HCCs showed at least a 2-fold reduction of the DLC-1 mRNA level when compared with that of the corresponding nontumorous livers. When the overall DLC-1 mRNA expression was compared between tumor and nontumorous liver groups, DLC-1 mRNA expression was reduced significantly in tumor (P < 0.0001, Wilcoxon test; Fig. 3A). Similar results were also obtained when β-actin was used as the endogenous control (data not shown).

Loss of Expression of DLC-1 mRNA Without Gene Deletion in Hepatoma Cell Lines. Of the 7 human carcinoma cell lines examined by semiquantitative RT-PCR and Northern blotting, 3 hepatoma cell lines (Bel-7402, SMMC-7721, and WRL) showed loss of expression of DLC-1 mRNA (Fig. 3, B and C). The DLC-1 gene was detected in all of these cell lines, except Bel-7402, which we showed previously to have gene deletion (27). Thus, loss of the DLC-1 expression in SMMC-7721 and WRL cell lines was not caused by gene deletion, and epigenetic changes such as hypermethylation of the DLC-1 promoter might be involved in DLC-1 gene silencing.

Methylation Status of DLC-1 CpG Island in Hepatoma Cell Lines. In silico analysis showed that the DNA sequence 5’ up-stream to the DLC-1 gene had a 689-bp CpG island, and its promoter activity has been demonstrated recently (33). Therefore, we screened the methylation status on this region in the hepatoma cell lines by methylation-sensitive restriction enzyme digestion with Smal or HpaII, followed by PCR amplification with primers designed to flank the CpG island of the DLC-1 gene. As controls, genomic DNA digested with MspI, which is a methylation-insensitive isozyme of HpaII, and undigested genomic DNA was also amplified at the same time. If the DLC-1 CpG island was not methylated, DNA template would be cleaved by Smal or HpaII, and no PCR product could be detected. On the other hand, these restriction sites could be blocked by DNA methylation and, hence, the DNA template was resistant to cleavage and allowed amplification by PCR. MSP product was detected in...
SMMC-7721 and WRL cells, which showed no expression of DLC-1 mRNA, after both Smal and HpaII digestion. This suggested that the DLC-1 CpG island was hypermethylated in these two cell lines. Interestingly, PLC/PRF/5 cells, which had DLC-1 mRNA expression, also exhibited a methylation-specific fragment after Smal digestion, although the intensity of this fragment was much weaker than that of the undigested control (Fig. 4A).

To additionally confirm the methylation status as well as the methylation profile of the DLC-1 CpG island in hepatoma cells, we performed bisulfite sequencing on at least four individual clones from SMMC-7721, HepG2, and PLC/PRF/5 cells. In agreement with the results obtained from methylation-sensitive restriction enzyme study, nearly all of the CpG dinucleotides from HepG2 cells were unmethylated, whereas SMMC-7721 cells showed extensive hypermethylation at these CpG dinucleotides. PLC/PRF/5 cells, on the other hand, showed complete methylation in two clones and remained unmethylated in another two clones (Fig. 4B). Therefore, PLC/PRF/5 cells likely have heterozygous methylation on the CpG island of the DLC-1 gene.

**DISCUSSION**

Hyperactivation of Rho family GTPases has been implicated in tumorigenesis. Recent studies have indicated that active Rho family proteins are essential for Ras oncoprotein, and dominant-negative mutants of RhoA, Rac1, and Cdc42 can block Ras transformation. In contrast, constitutively active RhoA, Rac1, and Cdc42 are oncogenic (35–38). Although mutations in Rho family genes are rare (39–42), overexpression of the Rho proteins has been detected in a variety of human tumors (39, 42–44). Furthermore, overexpression of RhoA is associated with more advanced histological grade, more advanced tumor stages, increased invasiveness, and a higher metastatic potential in human cancers (42, 44).

DLC-1 is a putative tumor suppressor gene, first reported by Yuan et al. (12). *In-silico* analysis has revealed that the COOH terminus of DLC-1 contains a highly conserved RhoGAP domain, which usually functions to catalyze the intrinsic GTPase activity of Rho family proteins (34). In this study, we have confirmed that the DLC-1-1 is a RhoGAP by *in vitro* GTPase activity assay. Thus far, 53 RhoGAP domain-containing proteins have been identified from database search (34). Most of them have GAP activity specific for RhoA, Rac1, or Cdc42, and possess GAP activity toward more than one member of the Rho family proteins. For instance, p190B was found to act on RhoA, Rac1, and Cdc42 (45). Our results indicate that DLC-1 possesses GAP activity, which is specific for RhoA and Cdc42. In the presence of DLC-1, the GTP hydrolysis activity of RhoA was dramatically increased, whereas DLC-1 had a less potent effect on Cdc42 and did not have effect on Rac1. We have identified recently a novel DLC-1 homology, named as DLC-2, at chromosome 13q12.3 (42). DLC-2 shares high identity with DLC-1 in amino acid sequence and also contains similar functional domains that have been found in DLC-1 (SAM, RhoGAP, and START domain). Interestingly, our previous data showed that the RhoGAP function was essential for DLC-2-mediated growth inhibition of Ras-induced transformation of NIH3T3 cells (42). The RhoGAP domain is the most conserved region...
between DLC-1 and DLC-2, suggesting that they may serve similar RhoGAP function. Indeed, in our previous and present studies, we have demonstrated that both DLC-1 and DLC-2 exert in vitro RhoGAP activity toward RhoA and Cdc42. These results imply that DLC-1 may serve as a tumor suppressor by negative regulation on the RhoA- and Cdc42-mediated transformation.

Our previous study showed that DLC-1 exerted inhibitory effects on the proliferation of hepatoma cells, and deletion of the DLC-1 gene was found in a subset of hepatoma cell lines and primary HCCs (27). These data suggested that loss of DLC-1 function might play a role in hepatocarcinogenesis. Inactivation of tumor suppressor genes requires “two-hits,” which can be a result of gene deletion, somatic mutations, and epigenetic changes such as hypermethylation, or any combination of these alterations. In the present study, we characterized the genetic and epigenetic changes on the DLC-1 gene in human HCCs. We first determined the deletion of the DLC-1 gene by LOH analysis using three microsatellite markers, of which D8S1754 is mapped inside the DLC-1 gene. Thus, allelic loss on this marker should directly represent heterozygous deletion of the DLC-1 gene. In a total of 100 HCC and nontumorous liver pairs, allelic losses on these three markers ranged from 44% to 50%. Our results suggest that allelic loss on the DLC-1 loci is very frequent, and heterozygous deletion may play an important role in DLC-1 inactivation in HCCs. Next, we determined whether somatic mutations on the DLC-1 gene contributed to the inactivation of DLC-1. Because DLC-1 is a RhoGAP, we hypothesized that somatic mutations on the DNA sequences encoding RhoGAP domain might affect its RhoGAP activity. For this reason, we sequenced the segment encoding RhoGAP domain in 60 HCC cases. The HCCs showed a significantly reduced level of DLC-1 mRNA in SMMC-7721 and WRL cells, and this demonstrated that the loss of DLC-1 mRNA expression was a consequence of DLC-1 CpG island hypermethylation.

In primary HCCs, we used MSP to screen the methylation status of DLC-1 CpG island. Our results revealed that hypermethylation on the CpG island of the DLC-1 gene was found in a significant proportion (24%) of the primary HCCs. Thus, DLC-1 inactivation by DNA methylation was not a rare event in primary HCC.

Taken together, our results suggest both genetic and epigenetic alteration contributing to the inactivation of the DLC-1 gene in primary HCC.

REFERENCES

CHARACTERIZATION OF DLC-1 GENE IN HCC


