PTEN/MMAC1 Mutation and Frequent Loss of Heterozygosity Identified in Chromosome 10q in a Subset of Hepatocellular Carcinomas

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Frequent allelic losses on chromosome 10q have been reported in several types of cancers, suggesting the presence of a putative tumor suppressor gene(s) on the chromosomal arm. We examined loss of heterozygosity (LOH) on chromosome 10q in 37 hepatocellular carcinomas (HCC) using eleven dinucleotide microsatellite markers, spanning the entire chromosome arm of 10q. Twelve (32%) out of 37 informative cases showed allelic losses of at least one locus on 10q and eight tumors showed a partial deletion of 10q. Analysis of deletion mapping of these eight cases identified two commonly deleted regions within the distal part of 10q (10q24-q26), a 20-cM interval flanked by D10S597 and D10S216 and a 24-cM interval flanked by D10S216 and D10S590. Moreover, we detected a somatic missense mutation (Met→Val) of a candidate tumor suppressor gene PTEN/MMAC1, located at 10q23.3, in one HCC with LOH of 10q. Our findings indicated the presence of putative tumor suppressor gene(s) in the distal region of 10q that might be involved in the development and progression of HCC. Inactivation of PTEN/MMAC1 gene located outside the commonly deleted region of 10q might also play an important role in a subset of HCCs.

Key words: PTEN/MMAC-1 — Hepatocellular carcinoma — LOH — Microsatellite marker

Loss of tumor suppressor gene(s) is involved in the development and progression of certain cancers. Recent studies have shown that alterations in several tumor suppressor genes and putative tumor suppressor loci are involved in development and progression of hepatocellular carcinoma (HCC), including p53 gene located on 17p13,1) RB1 on 13q14,2) 1p, 4q, 5q, 8p, 11p, 16q and 22q.3–9) Mutations of protooncogenes such as Ras and β-catenin have also been described, although the percentage of HCC showing such defects is small.10,11)

Frequent loss of heterozygosity (LOH) on chromosome 10q has been reported in various types of human cancers such as glioma, lung cancer, bladder cancer, non-Hodgkin lymphoma, malignant meningioma, melanoma, endometrial cancer, thyroid cancer, and prostate.12–21) These findings indicate that one or more putative tumor suppressor genes are located on this chromosomal arm.

A new putative tumor suppressor gene PTEN/MMAC1 was recently isolated from the chromosomal region 10q23.3 and somatic mutations of this gene have been reported in a variety of human cancers such as glioma, endometrial cancer, breast cancer, thyroid cancer, lung cancer, bladder cancer and prostate cancer.22–29) This gene was also identified as being responsible for two hamartoma syndromes; Cowden disease and Bannayan-Riley-Ruvalcaba syndrome.30–32)

Although frequent LOHs on 10q have been reported in HCC using a limited number of markers, chromosome 10q is among the least characterized in HCC.7,8) Therefore, we examined in the present study the involvement of PTEN/MMAC1 mapped to 10q23.3, in the progression of HCC.

Analysis of mutations of this new putative tumor suppressor gene in HCC DNAs was performed by single strand conformation polymorphism (SSCP) and direct sequencing analyses.

MATERIALS AND METHODS

Specimens and DNA extraction Thirty-seven pairs of tumors and corresponding non-cancerous tissues were obtained from 37 patients with HCC at the Department of Surgery II, Osaka University Medical School. All specimens had been previously examined histopathologically to confirm the diagnosis. Tumors were histologically classified as well differentiated, moderately differentiated, poorly differentiated, and undifferentiated carcinoma, according to the typing scheme of the Japanese Liver Cancer Society.33) After surgical removal, specimens were carefully dissected and stored at −80°C until extraction.
of DNA. High-molecular-weight genomic DNAs were extracted from frozen tissues according to the method described previously.\textsuperscript{24,25}

**LOH analysis using microsatellite markers** Ten primer sets for polymerase chain reaction (PCR) amplification of microsatellite markers were selected to span the whole chromosome arm according to the Centre d’Etude du Polymorphism Humain (CEPH)/Genethon linkage map.\textsuperscript{26} We also used D10S2491, a newly isolated microsatellite marker from the locus of PTEN/MMAC1.\textsuperscript{27} The linear order of CA-repeat microsatellite markers used is (centromere)–D10S561–D10S202–D10S2491–D10S564–D10S192–D10S597–D10S587–D10S216–D10S90–D10S212–D10S555–(telomere) (Table I). One of each pair of primer sets was labeled with $\gamma$-\textsuperscript{32}P\textsuperscript{-}ATP by using T4 polynucleotide kinase (Promega, Madison, WI). Genomic DNA (100 ng) was amplified by PCR in a 10-$\mu$l reaction mixture containing 1× PCR buffer (6.7 mM Tris, 16.6 mM ammonium sulfate, 6.7 mM EDTA, 10 mM $\beta$-mercaptoethanol), 20 pmol each of $\gamma$-\textsuperscript{32}P\textsuperscript{-}ATP-labeled primer and non-labeled primer, 1 U of Taq DNA polymerase, 0.8 mM dNTP mixtures, and 1.5 mM of MgCl\textsubscript{2}. Thirty-five PCR cycles were performed with each cycle consisting of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C in a Gene Amp PCR system 9600 (Perkin-Elmer Cetus, Norwalk, CT). Five microliter volume of each solution, mixed with an equal volume of loading dye (95% formamide, 0.02% xylene cyanol and 0.02% bromophenol blue), was denatured and then electrophoresed in 6% polyacrylamide gel containing 7.7 M urea and 32% formamide. Gels were dried and exposed to X-ray film for 4–48 h.

**Determination of LOH** Evaluation of LOH was performed by comparing the intensity of two alleles in informative cases using a scanning densitometer. Tumors were scored as exhibiting LOH if there was an absence or >50% reduction in intensity of one allele in the tumor sample compared to the normal allele.

**Mutation analysis** SSCP analysis was performed by PCR amplification of each of the nine exons, corresponding to the coding region of PTEN/MMAC1 gene. PCR primers were designed in introns to amplify each whole exon and intron-exon boundary. The nucleotide sequences of PCR primers have been described previously\textsuperscript{28} except primers for exon 5, which were designed as follows: forward primer, 5'-CTATCCTCTAGGTATTACTTTTTACC-3'; reverse primer, 5'-TCGAGATCCAGGAGAGGA-3'. PCR reactions were performed in 10-$\mu$l solutions containing 50 ng of genomic DNA, 2 pmol of each primer set, and 0.5 U of Taq I polymerase. SSCP analysis was performed using 5% non-denaturing polyacrylamide gels (acylamide:N, N-bisacrylamide 99:1) containing 10% glycerol. After electrophoresis for 16 h at 4°C, the gels were stained with Silver Green II (TaKaRa, Otsu) and scanned with a fluorescence image analyzer (FMBio II Multi-View, TaKaRa). PCR products showing a mobility shift in SSCP analysis were subjected to sequence analysis using a Direct Sequencing Kit (“BioDye,” PE Applied Biosystems, Warrington, UK).

**RESULTS**

**LOH analysis of HCC** DNAs from 37 HCCs were examined for LOH using a panel of eleven microsatellite markers spanning the whole region of chromosome 10q. Table I lists all dinucleotide repeat markers used for LOH analysis, as well as the frequency of LOH at each locus. The frequency of LOH was higher at the locus of the marker closer to the telomeric region of 10q. The frequency of LOH was highest at D10S212 and D10S590 (6 of 18, 33%), and lowest at D10S561 (1 of 14, 7%). Four out of 26 (15%) tumors showed LOH at the PTEN/MMAC1 locus (D10S2491). All 37 cases were informative with two or more markers and 12 (32%) showed allelic losses of at least one locus on 10q. Among 12 tumors with LOH, eight showed LOH at some loci, whereas the others showed retention of heterozygosity, indicating partial or interstitial deletions of the long arm of chromosome 10 in these tumors. Representative autoradiograms of cases with partial or interstitial deletions are shown in Fig. 1. Tumor biopsy #117 showed LOH at D10S212, but heterozygosity at D10S216 (Fig. 1a). Tumor biopsy #127 showed LOH at D10S587, but retained heterozygosity at D10S590 (Fig. 1b). Fig. 2 summarizes the results of LOH analysis with 11 markers in 12 cases with LOH. All samples showed LOH at the distal part of 10q. Two commonly deleted regions in the distal part were located in a 20-cM interval flanked by D10S597 and D10S216, and a 24-cM interval between D10S590 and D10S216, as shown by bars with arrows in Fig. 2.

**Table I. Frequency of Loss of Heterozygosity (LOH) at Different Microsatellite Loci on Chromosome 10q**

| Microsatellite marker | Informative cases | Cases with LOH | % of LOH |
|-----------------------|-------------------|----------------|---------|
| D10S561               | 14                | 1              | 7       |
| D10S202               | 19                | 2              | 11      |
| D10S2491              | 26                | 4              | 15      |
| D10S564               | 18                | 3              | 17      |
| D10S192               | 19                | 5              | 26      |
| D10S592               | 21                | 5              | 24      |
| D10S587               | 15                | 4              | 27      |
| D10S216               | 26                | 5              | 19      |
| D10S590               | 15                | 5              | 33      |
| D10S212               | 18                | 6              | 33      |
| D10S555               | 19                | 5              | 26      |
Tumor Suppressor Genes on 10q in HCC

Fig. 1. Autoradiograms of LOH analyses of hepatocellular tumors showing partial or interstitial deletions on chromosome 10q. a, tumor biopsy #117; b, tumor biopsy #127. Microsatellite markers are listed underneath each autoradiogram of paired DNA from HCC tumors (T) and corresponding normal tissues (N). Arrowheads indicate polymorphic bands.

Fig. 2. Deletion map of chromosome 10q in 12 HCC tumors showing LOH at different loci. Tumor biopsy numbers are shown above and names of markers on the left. A diagram of chromosome 10q is at the far left with locations and order of microsatellite markers indicated; distances between loci indicated are in centimorgans (cM). Open circles, retention of heterozygosity; solid circles, LOH; gaps between circles, uninformativeness for specific markers. The commonly deleted regions are indicated by arrows at the right.

Fig. 3. a: SSCP analysis of the PTEN gene in HCC tumors. The arrowhead indicates the abnormally shifted band in lane 3 (tumor biopsy #134). The presence of the shifted band was confirmed by a repeated experiment. b: Sequence analysis of the PTEN/MMAC1 gene PCR product showing the shifted band in SSCP analysis. The normal base pair of the sequence (A) was altered to (G) in tumor biopsy #134, resulting in substitution of GTG (Val) for ATG (Met). The sequence (A) remaining in tumor DNA may be produced by amplification of genomic DNA from normal cell contamination.
Clinicopathological data were available for 16 out of 37 HCC tumors. With respect to the grade of tumor differentiation, no LOH was detected in five moderately differentiated tumors, whereas six out of 11 poorly differentiated or undifferentiated HCC tumors showed LOH at loci on 10q.

**Mutation analysis of PTEN/MMAC1 in HCC** To examine whether PTEN/MMAC1 is a candidate tumor suppressor gene for HCC, all tumors showing LOH at any locus on chromosome 10q were subjected to mutation analysis of the PTEN/MMAC1 gene by SSCP and direct sequencing. One (tumor biopsy #134) out of 12 tumors showed an abnormally shifted band on SSCP analysis of exon 2. A somatic missense mutation (Met→Val) was detected at codon 35 in DNA sequence analysis of this tumor (Fig. 3, a and b).

**DISCUSSION**

We have presented here a detailed LOH analysis of chromosome 10q in 37 HCC tumors. Frequent LOHs (33%) were detected especially in the distal part of 10q at loci D10S212 and D10S590. Furthermore, analysis of deletion mapping defined two commonly deleted regions within the distal part of 10q (10q24-26). Recent studies have shown that the distal region of chromosome 10q is commonly deleted in glioma, lung cancer, melanoma, prostate cancer, bladder cancer, and thyroid tumor.

Recent studies have isolated a putative tumor suppressor gene, PTEN/MMAC1, from near the centromeric region of 10q (10q23.3). The PTEN/MMAC1-encoded protein exhibits a lipid phosphatase activity, which is thought to be responsible for its tumor suppressor function. Somatic mutations of this gene have been reported in various types of human cancers. The above findings suggest that there are plural tumor suppressor genes on chromosome 10q.

To examine further the involvement of the PTEN/MMAC1 gene in HCCs, we searched for mutations in 12 tumors showing LOH at loci on 10q. SSCP and mutation analysis detected a single somatic mutation (Met→Val) in exon 2 of PTEN/MMAC1 gene in advanced stage HCC tumor (Tumor Stage III, undifferentiated type). According to LOH analyses using a panel of eleven microsatellite markers, although allelic status was not informative at the locus of PTEN/MMAC1 (D10S2491), tumor biopsy #134 showed LOH at all loci of flanking informative markers. These results suggest a loss of one allele at the PTEN/MMAC1 locus, and that a mutation of this gene in the remaining allele occurred in the tumor DNA, resulting in inactivation of both alleles. Although the function of the mutated PTEN protein is not yet clear, a point mutation at codon 35 was previously reported in juvenile polyposis coli and was not observed in 93 controls. These facts indicate that the variant may be a tumor-associated change. In the present study, only four out of 26 informative cases (15%) showed LOH at D10S2491, isolated from the PTEN/MMAC1 locus, and the most frequent LOH was observed at the more distal part of 10q. These results indicated that plural tumor suppressor genes involved in hepatocarcinogenesis might exist on chromosome 10q.

In conclusion, we have obtained important new information on LOH in chromosome 10q and mutation of a putative tumor suppressor gene, PTEN/MMAC1, in a subset of HCC. Inactivation of PTEN/MMAC1 and unknown tumor suppressor gene(s), present in the distal region of 10q (10q24-q26), may play an important role in the progression of HCC.

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