PTEN/MMAC1 Mutations in Hepatocellular Carcinomas: Somatic Inactivation of Both Alleles in Tumors

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Allelic loss of loci on chromosome 10q occurs frequently in hepatocellular carcinomas. Somatic mutations of the PTEN/MMAC1 gene on this chromosome at 10q23 were recently identified in sporadic cancers of the uterus, brain, prostate and breast. To investigate the potential role of PTEN/MMAC1 gene in the genesis of hepatocellular carcinomas, we examined 96 tumors for allelic loss on 10q and also for subtle mutations anywhere within the coding region of PTEN/MMAC1 gene. Allelic loss was identified in 25 of the 89 (27%) tumors that were informative for polymorphic markers in the region. Somatic mutations were identified in five of those tumors: three frameshift mutations, a 1-bp insertion at codon 83–84 in exon 4 and two 4-bp deletions, both at codon 318–319 in exon 8; two C-to-G transversion mutation, both at −9 bp from the initiation codon in the 5′ non-coding region of exon 1. No missense mutation was observed in this panel of tumors. In most of the informative tumors carrying intragenic mutations of one allele, we were able to detect loss of heterozygosity as well. These findings suggest that two alleles of the PTEN/MMAC1 gene may be inactivated by a combination of intragenic point mutation on one allele and loss of chromosomal material on the other allele in some of these tumors.

Key words: PTEN/MMAC1 — Hepatocellular carcinoma — Tumor suppressor gene — Somatic mutation — Loss of heterozygosity

Hepatocellular carcinoma (HCC) is one of the most common human cancers in the world, with an annual incidence of 250,000 cases. Epidemiological studies have shown an association of chronic infection of hepatitis B virus (HBV) with HCCs. Other risk factors include chronic infection of hepatitis C virus (HCV), heavy alcohol intake, and exposure to aflatoxin B1. However, the role of hepatotropic viral agents and the molecular events leading to liver carcinogenesis remain unknown. A mutagenic role of HBV DNA integration in the host genome, that occurs frequently in the early stages of HBV-associated tumorigenesis, has been conclusively established only in rare cases,1, 2 suggesting the involvement of more indirect transformation pathways. A common feature in chronic viral hepatitis and liver cirrhosis is long lasting inflammation of the liver associated with chronic regenerative conditions, which might enhance the susceptibility of liver cells to genetic changes.

Inactivation of tumor-suppressor functions usually occurs as a consequence of mutation of one allele and loss of the other allele (loss of heterozygosity, LOH).3, 4 In HCCs, LOH has been reported on chromosome arms 1p, 4q, 5q, 6q, 8p, 10q, 11p, 16p, 16q 17p, and 22q.5–10 In particular, we and others have previously noted frequent LOH involving loci on chromosome arm 10q in HCCs.11–15

Analysis of homozygous deletions affecting chromosome 10q23 led to the identification of a new tumor suppressor gene in this region, designated PTEN/MMAC1.16, 17 Germline mutations of PTEN/MMAC1 are responsible for dominantly inherited neoplastic syndromes, Cowden disease and Bannayan-Zonana syndrome.18, 19 PTEN/MMAC1 encodes a 403-amino acid dual-specificity phosphatase containing a region of homology to tensin and auxillin, cytoskeletal proteins that interact with adhesion molecules.16, 17, 20 Somatic mutations of this gene have been detected in several tumor types including those of the uterus, brain, prostate, and the breast.16, 17, 21–23

To determine the role of PTEN/MMAC1 genetic alterations in the development and/or progression of HCCs, we examined this gene for mutation and allelic loss in 96 primary HCCs, and looked for correlations between PTEN/MMAC1 mutations and certain clinicopathological
parameters. Here we report evidence which suggests that both alleles of the PTEN/MMAC1 gene are inactivated in at least 5% of HCCs.

MATERIALS AND METHODS

Samples and DNA preparation Tumors and corresponding non-cancerous tissues were obtained from 96 patients who underwent surgery for primary HCCs. No metastases to other organs or distant lymph nodes were observed. All tissues were frozen immediately after surgery and stored at −80°C. DNA was extracted from frozen tissues by means of the procedures described previously.24)

The panel of clinico-pathological parameters studied included: tumor stage, serum hepatitis virus markers (HBsAg or HCV Ab), histologic type, tumor size, and pathological state of surrounding non-tumorous liver. The tumor stage for each case was determined according to the TNM classification. Histological grades of HCC were divided into three categories (well-differentiated, moderately differentiated, and poorly differentiated carcinoma), according to the typing scheme of the Japanese Liver Cancer Society (1992). These three groups correspond to grade I, II, and III+IV, respectively, of the Edmondson-Steiner classification.

LOH analysis Matched samples of normal and tumor genomic DNAs were analyzed for LOH with two microsatellite markers (D10S587 and D10S212) on the 10q arm near PTEN/MMAC1. Details of these markers were described in the CEPH/Genethon linkage map.25) Each polymerase chain reaction (PCR) was performed in a Gene Amp PCR system 9600 (Perkin-Elmer Cetus, Norwalk, CT) using 10 ng of template DNA in 10 µl volumes of mixture containing 1× PCR buffer (Boehringer Mannheim), 200 mM dNTPs, 2 pmol each primer, 2 mCi of [α-32P]dCTP, and 0.5 units of Taq polymerase. Cycling conditions were 30 cycles of 94°C for 30 s, 55−60°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min (Gene Amp PCR 9600 System). Each PCR product was mixed with 10 µl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol), heated at 94°C for 5 min, rapidly cooled on ice, and applied to a 5% polyacrylamide gel containing 5% glycerol in 0.5× TBE buffer. Electrophoresis was performed under two different conditions to improve resolution for detecting different types of SSCP variants: 150 V for 16 h at room temperature, and 240 V for 16 h at 4°C. Gels were dried and autoradiographed with intensifying screens. For sequence analysis, DNA eluted from variant bands resolved on PCR-SSCP gel was excised and used as a template for PCR amplification under conditions described elsewhere.27) Each of these PCR products was purified using SUPREC-02 (TaKaRa, Tokyo), according to the manufacturer’s instructions. Sequencing was performed with a [γ-32P]-end-labeled primer using the ThermoSequenase cycle sequencing kit (Amersham, Cleveland, OH), according to the manufacturer’s instructions. After electrophoresis at 1800 V for 2–3 h, gels were dried and exposed to X-ray film at room temperature for 16–24 h.

RESULTS

LOH analysis Two microsatellite markers D10S587 and D10S212 on the 10q arm, around the PTEN/MMAC1 gene, were used for LOH analysis of 96 cases. Eighty-nine patients in our study were informative, and LOH at one or both of the marker loci was detected in 25 of the tumors (27%).

PTEN/MMAC1 mutation PCR-SSCP screening of DNAs from the primary HCCs for mutations in the PTEN/MMAC1 gene itself detected aberrant bands in five cases; representative results are shown in Fig. 1. These cases were subsequently analyzed by direct sequencing; Fig. 2 shows representative results. The somatic C-to-G transversion at nucleotide position −9 from the initiation codon was identified in tumors 60 and 62; the 1-bp inser-
PTEN/MMAC1 Mutations in Hepatocellular Carcinoma

Table I. Sequences of PTEN/MMAC1 Primers Used for PCR-SSCP Analysis

| Exon | Sense primer (5′-3′) | Antisense primer (5′-3′) | cDNA sequence amplified in PCR |
|------|---------------------|--------------------------|-------------------------------|
| 1-a | GCCATCTCTCTCCCTTTTTT | AGGTCAAGTCTAAGTCAAGT | 1-U93051 1-79 79 |
| 1-b | AAAGAGAGTGGTAGAAGGAAAC | CTAAGAGAGTGGACAGAAAGGTA | 16-79 64 |
| 2-a | GATGCTGCTATCTCTGAGTAT | TCAATATTGGTCTCTGTATACGC | 80-152 73 |
| 2-b | ATCCCAAATTCATTGCTATGGA | ATGAATATAAACATCAATATTTGAAA | 86-164 79 |
| 3 | TTTTTGTTAATGTTGCTTTCCTT | TTAGAAGATTTGCTACACATC | 165-209 45 |
| 4 | GCAAAAGATACTTTATATACCTT | TCGGAGTTAGTTATACACATA | 210-253 44 |
| 5-a | AGTTTTTTTTCATTTGACTGAGGT | GGTCAAGATCTTACAAAAGG | 254-328 75 |
| 5-b | CTTTGAAGAAGGAAACCAAC | CCAGCTTTATAGTTGGAATTG | 266-380 115 |
| 5-c | GATCTGCTGCTATCTCTGAGT | TGGCCCGATGTAATGAAATATG | 319-431 113 |
| 5-d | TGTAAAGCTGGGAAAGG | TTCAGTCTCTACTTTCCCCT | 370-483 114 |
| 5-e | TCGGGGCAAAATTTTAAAGGC | TCAAGAAGGAGGAGAAACAG | 423-492 70 |
| 6-a | CATAGCAATTTAGTGAATAC | CAGGTAGCTATAATAATACACAT | 493-543 51 |
| 6-b | TTCTGTCCACCAGGGAGTAA | GTTTCAAACATCATCTTTCAGT | 493-605 113 |
| 6-c | CCTGTTAAAGAATCATCTTGGAT | GTTCTATACATGGGAAGGATG | 540-634 95 |
| 7-a | TGACAGTTGCTGACATGAAAG | GTGTCGGGGTTTCCGTTGAT | 635-700 66 |
| 7-b | TTGTCCATTCTCAGCTAAAG | ACTCTACTTGTATACACATC | 644-769 126 |
| 7-c | GACATGCTGCTATCTTGGAT | ACGAGAAGATTAAGTAGAAACCTTT | 706-801 96 |
| 7-d | GATATCAAAGTATAGGATCTTCC | GGAATTCTCTTTGTTAAGAAG | 754-801 48 |
| 8-a | TAATTTAAATGTTATCTTCCCTT | TCTCGAGTTTCCCTTGTGTC | 802-864 63 |
| 8-b | GAGAAAATGTTCCAATTTTGGATG | ACTGAATGCTATGCTTCTT | 802-915 114 |
| 8-c | GGAGACCTCGAGAAAAGTGAAGA | TGTTAAGAATGATGACATATTTC | 852-963 112 |
| 8-d | ATCGAGCAATTTGCTGATAG | TATCGGTTGCTTTGCTTT | 898-1007 110 |
| 8-e | GAATATCTAGTTACTTCTTCAACA | CACCAAGTCAAAAGAACCAATG | 940-1026 87 |
| 9-a | TACGATATATGCTGATGCTG | TACAGAAGGATGCACTGATCC | 1027-1095 69 |
| 9-b | CTGCACTGCTAGAACAACAGTAG | GTCAAGTTGGTTACAGATATC | 1033-1152 120 |
| 9-c | CTGCACTGACACTGATTTAGTG | TCAAGTGTGTATGACTGATCT | 1091-1203 113 |
| 9-d | CGAACCACTTCTGCTGAT | TTTTCTAGGTTTTCATACCC | 1139-1212 74 |

Fig. 1. PCR-SSCP analysis of the PTEN/MMAC1 gene exons in representative cases. Case numbers are shown at the top of each lane; arrows indicate an aberrant SSCP band identified in (A) exon 1 (5′-non-coding region), (B) exon 4, and (C) exon 8.
tion at codon 83/84 in tumor 54 caused a frameshift that created a new stop codon at codon 91; the 4 bp deletion at codon 318/319 in tumors 34 and 43 resulted in a frame-shift that created a new stop codon at codon 321. Table II lists the somatic mutations identified among the tumors in our panel. All five mutations were present in tumor DNA, but not in corresponding normal DNA from the same patients. These mutations were not observed in 192 chromosomes derived from the general Japanese population. No significant association was observed between the mutations in the \textit{PTEN/MMAC1} gene and any clinico-pathological parameter.

Two-hit inactivation of the \textit{PTEN/MMAC1} gene We examined the allelic status of the five primary HCCs in which a subtle mutation was identified through the LOH analysis. In three of the four tumors informative for either marker, we were able to detect loss of an allele (tumors 43, 54 and 62; Table I). These findings, together with the detection of mutation on one allele, suggest that two alleles of the \textit{PTEN/MMAC1} gene may be inactivated by a combination of intragenic point mutation on one allele and loss of chromosomal material on the other allele in most of these tumors.

**DISCUSSION**

The \textit{PTEN/MMAC1} gene, which lies at 10q23, has been identified as a predisposing gene for two dominantly inherited neoplastic syndromes. Germline mutations of \textit{PTEN/MMAC1} were found in Cowden disease and Bannayan-Zonana syndromes. Somatic mutations in \textit{PTEN/MMAC1} have been described in uterine, brain, prostate, and breast cancers.16–23) The present study represents the first extensive mutational screening of this gene in tumor DNA of primary HCCs. We identified five mutations in HCCs, all of which were somatic mutations.

The sensitivity of SSCP analysis that we utilized for mutation detection depends on the length and the primary structure of the fragment to be analyzed and on the conditions chosen for electrophoresis. Although we designed PCR primers so that each amplicon falls within a short fragment size range (102–128 bp) to achieve higher sensitivity, it is known that certain DNA variations are undetectable by SSCP under some electrophoretic conditions. Thus, though we carried out our electrophoretic experiments under two different sets of conditions, some subtle mutations might have escaped detection by our SSCP...
method. The apparent discrepancy between the rate of LOH on 10q23 and the rate of subtle mutation within the PTEN/MMAC1 gene may be partly explained by such incompleteness of detection. We screened the complete coding sequence of the PTEN/MMAC1 gene by SSCP, but not the upstream promoter regions, introns, and the 3' non-coding regions. Any subtle mutations present in those regions would have escaped our SSCP screening, and this may also partly explain the apparent discrepancy between the rates.

In previous reports that analyzed brain, prostate, breast and endometrial tumors, as well as inherited neoplastic syndromes, the majority of PTEN/MMAC1 mutations were also detected in exons 5, 7 and 8.16-21 Of those, most of the missense mutations were identified in exon 5, encoding the putative phosphatase domain, and exons 7 and 8, encoding a potential tyrosine kinase phosphorylation site, which are essential for the function of the PTEN/MMAC1 protein. In the present study of primary HCCs, mutations were detected in three of the nine exons; exons 1, 4 and 8. In addition, in our series of HCCs, no missense mutation was detected in the PTEN/MMAC1 gene, whereas a somatic mutation at -9 in the 5' non-coding region was identified in two tumors. Although the functional significance of this mutation, whether transcriptional or post-translational, remains to be elucidated, the specificity in mutation pattern may be a characteristic feature of PTEN/MMAC1 mutation in HCCs.

Our study revealed LOH in 25 (27%) of the 89 informative tumors. In the majority of the tumors in which we identified point mutations of the PTEN/MMAC1 gene, we detected allelic loss with markers surrounding the PTEN/MMAC1 locus. PTEN/MMAC1 encodes a 403-amino acid dual-specificity phosphatase containing a region of homology to tensin and auxillin, cytoskeletal proteins that interact with adhesion molecules. Thus, deletion of this locus might profoundly alter the growth of hepatocytes. Germline mutations of PTEN/MMAC1 gene are responsible for Cowden disease and Bannayan-Zonana syndrome, in which elevated risk of breast and thyroid cancers is noted in the affected family members. Cowden disease is not associated with elevated risk of hepatocellular carcinoma. However, results presented here indicate that somatic PTEN/MMAC1 mutations are present in a portion of hepatocellular carcinomas. We suggest that the PTEN/MMAC1 gene is altered in a portion of hepatocellular carcinomas and that inactivation of this gene occurs during the course of hepatocellular carcinogenesis.

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REFERENCES

1) Dejean, A., Bouguerelet, L., Grzeschik, K. H. and Tiollais, P. Hepatitis B virus DNA integration in a sequence homologous to v-erb-A and steroid receptor genes in a hepatocellular carcinoma. Nature, 322, 70–72 (1986).
2) Wang, J., Chenivesse, X., Henglein, B. and Bréchot, C. Hepatitis B virus integration in a cyclin A gene in a hepatocellular carcinoma. Nature, 343, 555–557 (1990).
3) Knudson, A. G. Mutation and cancer: statistical study of retinoblastoma. Proc. Natl. Acad. Sci. USA, 68, 820–823 (1971).
4) Knudson, A. G. Antioncogenes and human cancer. Proc. Natl. Acad. Sci. USA, 90, 10914–10921 (1993).
5) Wang, H. P. and Rogler, C. E. Deletions in human chromosome arms 11p and 13q in primary hepatocellular carcinomas. Cytogenet. Cell Genet., 48, 72–78 (1988).
6) Buetow, K. H., Murray, J., Israel, J., London, W., Smith, M., Kew, M., Blanquet, V., Bréchot, C., Redeker, A. and Govindarajah, S. Loss of heterozygosity suggests tumor suppressor gene responsible for primary hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA, 86, 8852–8856 (1989).
7) Tsuda, H., Zhang, W., Shimosato, Y., Yokota, J., Terada, M., Sugimura, T., Miyamura, T. and Hirohashi, S. Allele loss on chromosome 16 associated with progression of human hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA, 87, 6791–6794 (1990).
8) Emi, M., Fujiwara, Y., Nakajima, T., Tsuchiya, E., Tsuda, H., Hirohashi, S., Maeda, Y., Tsuruta, K., Miyaki, M. and Nakamura, Y. Frequent loss of heterozygosity for loci on chromosome 8p in hepatocellular carcinoma, colorectal cancer, and lung cancer. Cancer Res., 52, 5368–5372 (1992).
9) Yeh, S. H., Chen, P. J., Chen, H. L., Wang, C. C. and Chen, D. S. Frequent genetic alterations at the distal region of chromosome 1p in human hepatocellular carcinomas. Cancer Res., 54, 4188–4192 (1994).
10) De Souza, A. T., Hankins, G. R., Washington, M. K., Orton, T. C. and Jirtle, R. L. M6P/IGF2R gene is mutated in human hepatocellular carcinomas with loss of heterozygosity. Nat. Genet., 11, 447–449 (1995).
11) Fujimori, M., Tokino, T., Hino, O., Kitagawa, T., Imamura, T., Okamoto, E., Mitsuobu, M., Ishikawa, T., Nakagama, H., Harada, H., Yagura, M., Matsubara, K. and Nakamura, Y. Allelotype study of primary hepatocellular carcinoma.
12) Nagai, H., Pineau, P., Tiollais, P., Baendia, M. A. and Dejean, A. Comprehensive allelotyping of human hepatocellular carcinoma. Oncogene, 14, 2927–2933 (1997).
13) Takahashi, K., Kudo, J., Ishibashi, H., Hirata, Y. and Niho, Y. Frequent loss of heterozygosity on chromosome 22 in hepatocellular carcinoma. Hepatology, 17, 794–799 (1993).
14) Yumoto, Y., Hanafusa, T., Hada, H., Morita, T., Ooguchi, S., Shinji, N., Mitani, T., Hamaya, K., Koide, N. and Tsuji, T. Loss of heterozygosity and analysis of mutation of p53 in hepatocellular carcinoma. J. Gastroenterol. Hepatol., 10, 179–185 (1995).
15) Piao, Z., Park, C., Park, J. H. and Kim, H. Allelotype analysis of hepatocellular carcinoma. Int. J. Cancer, 75, 29–33 (1998).
16) Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bignier, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H. and Parsons, R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science, 275, 1943–1947 (1997).
17) Steck, P. A., Pershouse, M. A., Jasser, S. A., Yang, W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D. H. and Tavtigian, S. V. Identification of a candidate tumor suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat. Genet., 15, 356–362 (1997).
18) Liaw, D., Marsh, D. J., Li, J., Dahia, P. L., Wang, S. I., Zheng, Z., Bose, S., Call, K. M., Tsou, H. C., Peacocke, M., Eng, C. and Parsons, R. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat. Genet., 16, 64–67 (1997).
19) Marsh, D. J., Dahia, P. L., Zheng, Z., Liaw, D., Parsons, R., Gorlin, R. J. and Eng, C. Germline mutations in PTEN are present in Bannayan-Zonana syndrome. Nat. Genet., 16, 333–334 (1997).
20) Myers, M. P., Stolarov, J. P., Eng, C., Li, J., Wang, S. I., Wigler, M. H., Parsons, R. and Tonks, N. K. P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. Proc. Natl. Acad. Sci. USA, 94, 9052–9057 (1997).
21) Risinger, J. I., Hayes, A. K., Berchuck, A. and Barrett, J. C. PTEN/MMAC1 mutations in endometrial cancers. Cancer Res., 57, 4736–4738 (1997).
22) Rhei, E., Kang, L., Bogomolnyi, F., Federici, M. G., Borgen, P. I. and Boyd, J. Mutation analysis of the putative tumor suppressor gene PTEN/MMAC1 in primary breast carcinomas. Cancer Res., 57, 3657–3659 (1997).
23) Kurose, K., Bando, K., Fukino, K., Sugisaki, Y., Araki, T. and Emi, M. Somatic mutations of the PTEN/MMAC1 gene in fifteen Japanese endometrial cancers: evidence for inactivation of both alleles. Jpn. J. Cancer Res., 89, 842–848 (1998).
24) Sato, T., Tanigami, A., Yamakawa, K., Akiyama, F., Kasumi, F., Sakamoto, G. and Nakamura, Y. Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. Cancer Res., 50, 7184–7189 (1990).
25) Gyapay, G., Morissette, J., Vignal, A., Dib, C., Fizames, C., Millasseau, P., Marc, S., Bernardi, G., Lathrop, M. and Weissenbach, J. The 1993–1994 Genethon human genetic linkage map. Nat. Genet., 7, 246–339 (1994).
26) Iida, A., Isobe, R., Yoshimoto, M., Kasumi, F., Nakamura, Y. and Emi, M. Localization of a breast cancer tumor suppressor gene to a 3-cM interval within chromosomal region 16q22. Br. J. Cancer, 75, 264–267 (1997).
27) Hirayama, T., Yamaki, E., Hata, A., Tsuji, M., Hashimoto, K., Yamamoto, M. and Emi, M. Five familial hypercholesterolemic kindreds in Japan with novel mutations of the LDL receptor gene. J. Hum. Genet., 43, 250–254 (1998).