

Infrequent Mutations in the *PTEN/MMAC1* Gene among Primary Breast Cancers

Kazuki Ueda,^{1,2} Misae Nishijima,¹ Hiroki Inui,² Masahiro Watatani,² Eiji Yayoi,^{3,6} Jun Okamura,³ Masayuki Yasutomi,² Yusuke Nakamura^{1,4} and Yasuo Miyoshi^{1,5}

¹Division of Clinical Genetics, Department of Medical Genetics, Biomedical Research Center, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565, ²First Department of Surgery, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589, ³Osaka Teishin Hospital, 2-6-40 Karasugatsuji, Tennouji-ku, Osaka 543 and ⁴Laboratory of Molecular Medicine, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108

Recently *PTEN/MMAC1*, a candidate tumor suppressor gene, was isolated from chromosome 10q23-24 and somatic mutations of this gene were detected in several malignancies including brain, prostate, and breast tumors. To investigate further the potential role of this gene in mammary carcinogenesis, we examined 69 primary breast cancers for mutations in *PTEN/MMAC1* by means of polymerase chain reaction single-strand conformation polymorphism and sequencing analysis. We detected only one somatic missense mutation, a change from T to C at codon 59 (TCA to CCA) resulting in substitution of Pro for Ser in the predicted protein. This site is located outside of phosphatase or phosphate-acceptor motifs, but this codon encodes a residue that is conserved in homologous proteins, tensin and auxilin and is likely to be crucial for normal function of *PTEN/MMAC1*. Among the 69 tumors examined, three low-frequency polymorphisms were found as well, one in the non-coding region of exon 1 and one each in introns 2 and 7. Our results suggested that mutation of the *PTEN/MMAC1* gene is not a major factor in the development of most primary breast cancers.

Key words: *PTEN/MMAC1* gene — Breast cancer — Chromosome 10q — Somatic mutation

Frequent LOH at chromosome 10q22-25 has been observed in a variety of cell lines or primary cancers from brain (40–75%),^{1–3} prostate (20–36%),^{4–7} breast (12–34%),^{1,8,9} and kidney (40%).¹ Recently *PTEN*, also known as *MMAC1*, was isolated from 10q23.3.^{1,9} According to the cited reports, somatic mutations of this gene, including homozygous deletions, were identified in nearly half of glioblastoma cell lines and in one-fourth of primary glial tumors examined, indicating that loss of *PTEN/MMAC1* function is probably associated with progression of glioblastomas. Furthermore, germ-line mutations of the *PTEN/MMAC1* gene have been found in patients with Cowden disease, an autosomal dominant syndrome carrying an elevated risk for cancers of the breast and thyroid.^{10–12} These findings suggested that *PTEN/MMAC1* gene mutations might contribute to the development or progression of sporadic primary breast cancers. In fact, mutations of this gene were reported in two of 26 breast cancer cell lines and in two of 14 primary breast tumors examined.^{1,9} To evaluate the importance of *PTEN/MMAC1* mutation(s) in mammary carcinogenesis, we undertook mutational screening of the entire coding region of the gene in 69 primary breast

cancers, by means of PCR-SSCP analyses and DNA sequencing.

MATERIALS AND METHODS

Tumor samples and preparation of genomic DNA

Tissue specimens were obtained from 69 patients undergoing surgery for breast cancer at the Cancer Institute Hospital (Tokyo) or Osaka Teishin Hospital (Osaka). Excised tissues were snap-frozen in liquid nitrogen and stored at –80°C until DNA extraction. The median age of patients at the time of surgery was 51 years (range of 26–81 years). According to the TNM classification of the Japanese Breast Cancer Society,¹³ the clinical stages found among these cases were as follows: stage I, 20 tumors; stage II, 27 tumors; stage III, 19 tumors; and unclassified, three tumors. Pathological diagnosis revealed that 10 were papillotubular carcinomas, 19 were solid-tubular carcinomas, 22 were scirrhous carcinomas, 10 were invasive lobular carcinomas, one was mucinous carcinoma, one was apocrine carcinoma, and six were unknown. Genomic DNA was extracted from the frozen tissues as described elsewhere.¹⁴

PCR-SSCP analysis Intronic primers (Table I) were designed to achieve amplification throughout the coding region (9 exons) of *PTEN/MMAC1*. Each 20- μ l PCR reaction mixture contained 50 ng of genomic DNA, 2 μ l of 10 \times PCR buffer, 250 μ M of each dNTP, 0.5 μ M of each primer, and 1 unit of Taq polymerase (TaKaRa, Otsu). Amplification was conducted for 35 cycles of 94°C for 30 s, 54–58°C for 30 s, and 72°C for 30 s in a thermal cycler

⁵To whom correspondence should be addressed.

⁶Present address: Kaizuka City Hospital, 3-10-20 Hori, Kaizuka, Osaka 597.

The abbreviations used are: LOH, loss of heterozygosity; *PTEN*, phosphatase and tensin homolog deleted on chromosome ten; *MMAC1*, mutated in multiple advanced cancers 1; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.

Table I. Primers for SSCP Analysis of *PTEN/MMAC1*

Exon	Forward	Reverse
1	TCCAGAGCCATTTCCATCC	CGTTCTAAGAGAGTGACAGA
2	AGTTTGATTGCTGCATATTTTC	CTGTGGCTTAGAAATCTTTTC
3	TCTTGATAGTATTAATGTAATTTCA	TGTATCTTTAACTCTACCTCA
4	GATTCAGGCAATGTTTGTAGTA	TAATCTGGATGACTCATTATTGT
5	CTTGTTAATTAATAAATTCAAGAGT	TCTCAGATCCAGGAAGAGG
6	TTAGTGAAATAACTATAATGGAA	CTGTTCCAATACATGGAAGG
7	CAGTTAAAGGCATTTCTGTG	GCTTTTAATCTGTCCTTATTTTG
8	ACAAAATGTTTCACTTTTGGGTA ^{a)}	ATCACATACATACAAGTCAACA
9	AAGATGAGTCATATTTGTGGGT	ATTCAAGTTTATTTTCATGGTGTT

a) This primer sequence was described by Steck *et al.*¹⁾

(Perkin Elmer-Cetus 9600, Norwalk, CT). The PCR products were resolved in 2% agarose gels and visualized by staining with ethidium bromide.

Non-radioisotopic SSCP analysis was performed as previously described.¹⁵⁾ A 2.5 μ l aliquot of each PCR product and 3.0 μ l of denaturing stop solution (95% formamide, 10 mM EDTA, 0.25% bromophenol blue and xylene cyanol) were mixed, heated to 95°C for 5 min and quenched on ice. A 2.5- μ l aliquot of each denatured mixture was loaded on a non-denaturing polyacrylamide gel (5% polyacrylamide gel containing 10% glycerol) and electrophoresed under running conditions of 500 V for 12 h at 4°C. After electrophoresis, gels were stained with "SYBR" Green II (FMC BioProducts, Rockland, ME) and visualized by using an FMBIO II Multi-View fluorescent image analyzer (TaKaRa).

DNA sequencing PCR products that revealed mobility shifts on SSCP analysis were cut from the gels and recovered. Re-amplification by PCR was followed by subcloning into pBluescript II SK(-) vector (Stratagene, La Jolla, CA) and sequencing using T3 or T7 primers. The sequencing analysis for each product was performed with an ABI 373S or 377 DNA automated sequencer (Perkin Elmer-Cetus) and the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Forester, CA).

RESULTS

Among the 69 tumor DNAs examined, we detected aberrant SSCP patterns in five (T10, T41, T55, T56, and T63; e.g., Fig. 1). We subsequently cloned and sequenced all five PCR products to characterize their nucleotide alterations. DNA sequencing of the product corresponding to the aberrant SSCP band derived from T55 revealed a T-to-C transition at codon 59 (TCA to CCA) that would result in a substitution of Pro for Ser in the encoded protein (Fig. 2A). To determine whether this DNA substitution had occurred as a somatic event and/or whether the normal allele was lost in the tumor, we took advantage of the creation of

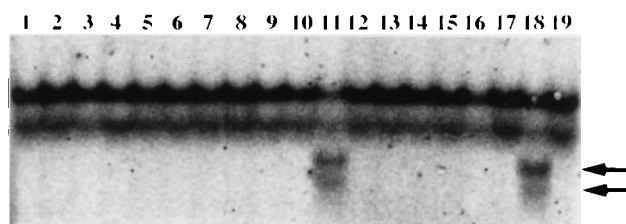


Fig. 1. SSCP analysis of exon 1 in the *PTEN/MMAC1* gene. Lanes 1–18 contain DNAs from breast cancer tissues and lane 19 corresponds to a normal control. Bands revealing mobility shifts in lane 11 (T41) and lane 18 (T63) are indicated by arrows.

a recognition site for *Bam*HI by this nucleotide alteration. Fig. 2B shows the result of electrophoresis after digestion of the PCR products of the tumor and corresponding normal tissue. The results clearly demonstrated nearly equivalent quantities of digested (136-bp and 88-bp) and undigested (224-bp) fragments. However, if we take account of the infiltration of normal cells, it is considered likely that the normal allele is lost in the tumor cells of T55 and that the DNA substitution had occurred as a somatic event.

Nucleotide changes in the remaining four cases were also characterized, but none of them was thought likely to affect the function of the gene product: in tumors T41 and T63, we confirmed a C-to-T substitution 9-bp upstream from the initial methionine codon of exon 1; in T10, an A-to-G alteration 60-bp downstream from the 5' end of intron 2; and in T56, an A-to-G change 6-bp downstream from the 5' end of intron 7. The change in T56 was confirmed to be a germ-line event by the presence of the same aberrant SSCP pattern in the normal tissue of this patient. We were unable to distinguish whether the remaining two types of change were somatic events or germ-line variations because no normal tissues were available from those three patients. Instead, we examined DNAs from 50 unrelated

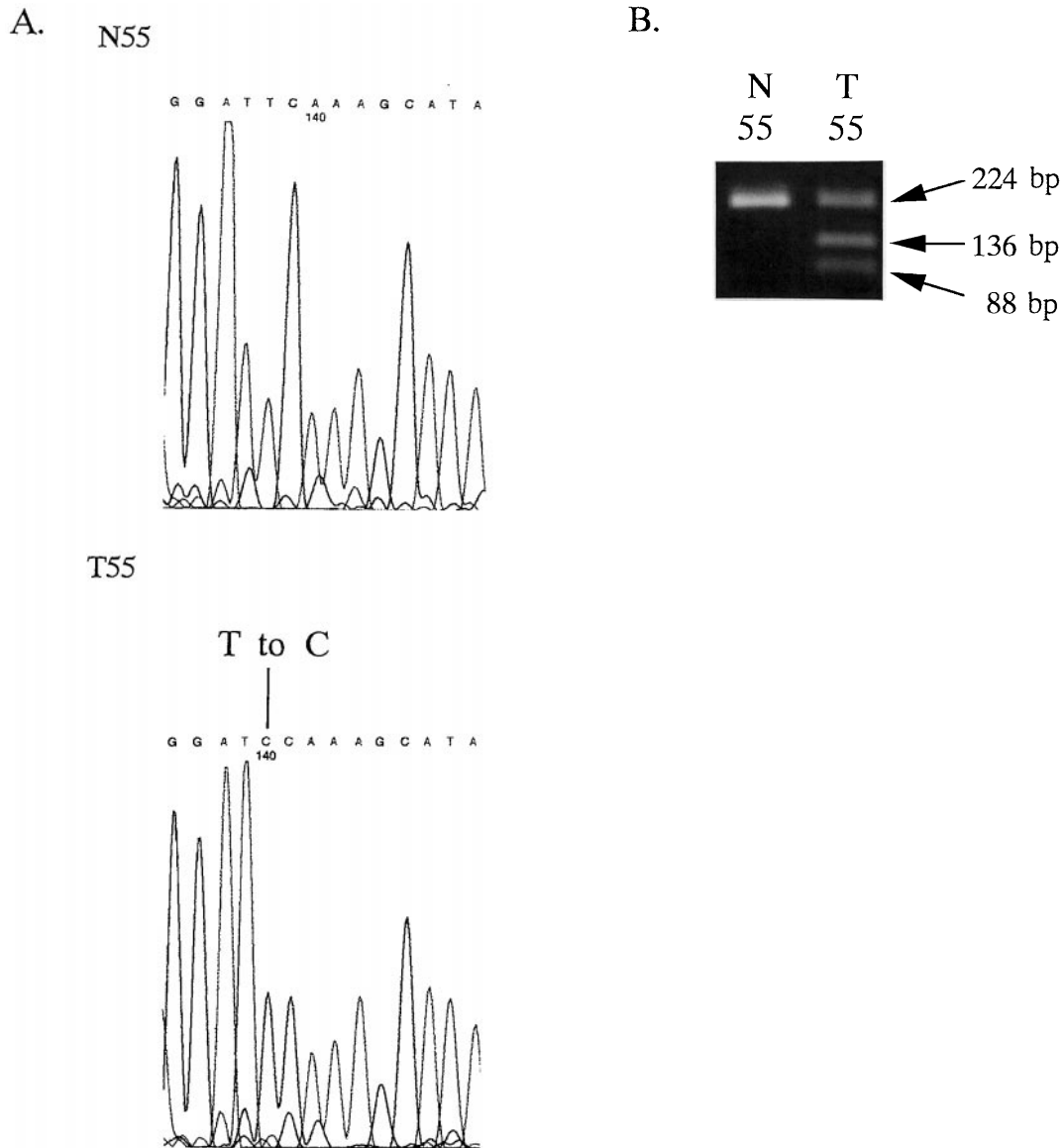


Fig. 2. A, Sequence analysis of the aberrant band recognized in tumor 55. A nucleotide substitution from T to C is apparent. B, Electrophoresis of PCR products amplified from constitutional genomic DNA of patient 55 (N55) and T55 after *Bam*HI digestion. In N55, an undigested 224-bp fragment is recognized and in T55, both undigested (224-bp) and digested (136-bp and 88-bp) fragments are present.

Japanese volunteers for the presence or absence of these specific alterations. Each of the two changes was observed at 4% frequency among the 100 alleles tested (data not shown), indicating that these substitutions are low-frequency polymorphisms.

DISCUSSION

PTEN/MMAC1 encodes a protein of 403 amino acids that includes a phosphatase core motif and two potential ty-

rosine phosphate acceptor motifs; this product shows sequence homology with tensin and auxilin, both of which are cytoskeletal proteins.^{1,9)} *PTEN/MMAC1* is assumed to possess tumor-suppressive activity through the removal of phosphate from tyrosine residues in tyrosine kinase oncoproteins that mediate growth signaling. Furthermore, on the basis of its homology with tensin, which can bind to actin filaments at focal adhesions, it may also participate in maintenance of cellular structure and regulation of cell growth, invasion and metastasis.^{1,9)}

Mutations in the *PTEN/MMAC1* gene have been reported in cancer cell lines and in 10 of 22 gliomas and two of four prostate cancers examined.^{1,9)} As mutation seems to be more frequent in glioma cell lines than in primary gliomas, such alterations may be associated with progression of glioma. Somatic mutations of the *PTEN/MMAC1* gene in breast and kidney cancers have also been reported, although at lower frequencies.^{1,9)} Although the somatic mutations identified so far in cell lines or primary tumors have been scattered among all exons except exon 9, substitutions or deletions of amino acid(s) preferentially occur in exons 5, 7, and 8 where phosphatase or phosphate-acceptor motifs are present, or at specific residues in exons 2 and 3 that are conserved in tensin and auxilin.^{1,9)} Missense germline mutations of *PTEN/MMAC1* gene identified in Cowden disease are more concentrated at codons within exons 5 and 7 that are responsible for phosphate-acceptor motifs.¹⁰⁻¹²⁾

In this study, we detected a missense mutation at codon 59 in T55, which is located outside of those motifs. However, this codon encodes a residue that is conserved in tensin and auxilin. As somatic mutations have been reported at codon 57, also encoding a residue conserved among these proteins,^{1,9)} both conserved amino acids are likely to be crucial for normal function of *PTEN/MMAC1*. The clinicopathological features of case 55 were as follows; the tumor was stage II (diameter 28 mm) of the TNM

classification and diagnosed as scirrhous carcinoma, and the patient died in 18 months after surgery (the cause of death was unclear). The higher frequency of deleterious mutations found in previous studies as compared with ours may reflect preselection of breast tumors on the basis of LOH on chromosome 10q.¹⁾ When this bias is accounted for, the frequencies of reported *PTEN/MMAC1* mutations in primary breast cancers are not significantly different.^{1,9)}

However, the fact that we found only one mutation among 69 primary breast cancers seems to indicate that somatic mutation in the *PTEN/MMAC1* gene itself is very rare in breast cancer and unlikely to play a significant role in mammary carcinogenesis. Still, we cannot exclude the possibilities that mutations may have occurred in the promoter region or in introns outside the searched region that affect transcription, or that intragenic deletions have been overlooked.

ACKNOWLEDGMENTS

This work was supported in part by a special grant for Strategic Advanced Research on Cancer from the Ministry of Education, Culture, Sports, and Science of Japan; and a "Research for the Future" Program Grant (96L00102) of the Japan Society for the Promotion of Science.

(Received September 8, 1997 / Revised October 20, 1997 / Accepted November 4, 1997)

REFERENCES

- 1) Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, A. 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. F. 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).
- 2) Rasheed, B. K., McLendon, R. E., Herndon, J. E., Friedman, H. S., Friedman, A. H., Bigner, D. D. and Bigner, S. H. Alterations of the TP53 gene in human gliomas. *Cancer Res.*, **54**, 1324-1330 (1994).
- 3) Bigner, S. H., Mark, J., Burger, P. C., Mahaley, M. S., Jr., Bullard, D. E., Muhlbaier, L. H. and Bigner, D. D. Specific chromosomal abnormalities in malignant human gliomas. *Cancer Res.*, **48**, 405-411 (1988).
- 4) Ittmann, M. M. Loss of heterozygosity on chromosomes 10 and 17 in clinically localized carcinoma. *Prostate*, **28**, 275-281 (1996).
- 5) Latil, A., Baron, J. C., Cussenot, O., Fournier, G., Soussi, T., Boccon, G. L., Le, D. A., Rouesse, J. and Lidereau, R. Genetic alterations in localized prostate cancer: identification of a common region of deletion on chromosome 18q. *Genes Chrom. Cancer*, **11**, 119-125 (1994).
- 6) Massenkeil, G., Oberhuber, H., Hailemariam, S., Sulser, T., Diener, P. A., Bannwart, F., Schafer, R. and Schwarte, W. I. p53 mutations and loss of heterozygosity on chromosomes 8p, 16q, 17p, and 18q are confined to advanced prostate cancer. *Anticancer Res.*, **14**, 2785-2790 (1994).
- 7) Deubler, D. A., Williams, B. J., Zhu, X. L., Steele, M. R., Rohr, L. R., Jensen, J. C., Stephenson, R. A., Changus, J. E., Miller, G. J., Becich, M. J. and Brothman, A. R. Allelic loss detected on chromosomes 8, 10, and 17 by fluorescence in situ hybridization using single-copy P1 probes on isolated nuclei from paraffin-embedded prostate tumors. *Am. J. Pathol.*, **150**, 841-850 (1997).
- 8) Sourvinos, G., Kiaris, H., Tsikkinis, A., Vassilaros, S. and Spandidos, D. A. Microsatellite instability and loss of heterozygosity in primary breast tumours. *Tumour Biol.*, **18**, 157-166 (1997).
- 9) Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, 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).
- 10) Nelen, M. R., Staveren, W. C. G., Peeters, E. A. J., Hassel, M. B., Gorlin, R. J., Hamm, H., Lindboe, C. F., Fryns, J.-P., Sijmons, R. H., Woods, D. G., Mariman, E. C. M., Padberg, G. W. and Kremer, H. Germline mutations in the PTEN/

- MMAC1 gene in patients with Cowden disease. *Hum. Mol. Genet.*, **6**, 1383–1387 (1997).
- 11) Liaw, D., Marsh, D. J., Li, J., Dahia, P. L. M., 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).
 - 12) Marsh, D. J., Dahia, P. L. M., 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).
 - 13) The Japanese Breast Cancer Society. “General Rules for Clinical and Pathological Recording of Breast Cancer,” 12th Ed. (1996). Kanehara Co., Tokyo.
 - 14) 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).
 - 15) Oto, M., Miyake, S. and Yuasa, Y. Optimization of nonradioisotopic single strand conformation polymorphism analysis with a conventional minislabs gel electrophoresis apparatus. *Anal. Biochem.*, **213**, 19–22 (1993).

Note Added in Proof: Similar findings were reported by others after submission of our manuscript (*Cancer Res.*, **57**, 3657–3659 (1997)).