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Infrequent Genetic Alterations of the *PTEN/MMAC1* Gene in Japanese Patients with Primary Cancers of the Breast, Lung, Pancreas, Kidney, and Ovary

Akira Sakurada,[1,4#] Akihiko Suzuki,[1,2#] Masami Sato,[1,4] Hiromitsu Yamakawa,[1] Kazuhiko Orikasa,[1] Shinji Uyeno,[3] Tetsuya Ono,[3] Noriaki Ohuchi,[2] Shigefumi Fujimura[4] and Akira Horii[1,5]

Departments of [1]Molecular Pathology, [2]Surgery II, [3]Radiation Research, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-77 and [4]Department of Thoracic Surgery, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-77

In the present study, we searched for genetic alterations of the entire coding region of *PTEN/MMAC1*, a recently isolated candidate tumor suppressor gene, in 178 specimens from Japanese patients with various malignant tumors by the polymerase chain reaction-single strand conformation polymorphism method. The samples consisted of 11 glioblastoma multiformes (GBMs), 14 astrocytomas, 47 breast cancers, 25 non-small cell lung cancers, 9 small cell lung cancers, 8 pancreatic cancers, 24 renal cell carcinomas, 20 ovarian cancers, and 20 metastatic lung tumors from various organs. Only one somatic frameshift mutation at codon 319 was observed in one (9%) of eleven GBMs. Our results suggest that mutation of the *PTEN/MMAC1* gene does not play a major role in carcinogenesis, at least in the tumor types from Japanese patients analyzed in this study.

Key words: Glioblastoma multiforme — Somatic mutation — *PTEN/MMAC1* gene

Gliomas are the most common primary brain tumors of the adult central nervous system in the U. S.,[1)] and the second most common in Japan.[2)] GBM is one of the brain tumors with a very poor prognosis: despite a variety of trials of surgical treatments and adjuvant therapies, the survival rate has not shown any significant improvement. To establish a method for better clinical management of patients with GBMs, it is necessary to understand the mechanisms of carcinogenesis. To date, several genetic alterations, including amplification and rearrangement of the epidermal growth factor receptor gene[3)] and allelic deletions of 1p, 9p, 10, 13q, 17p, 18q, 19q, and 22q, have been reported in GBMs (reviewed by Louis and Gussela[4)]); among these, loss of chromosome 10 was the most frequently observed.[4)] Recently, a candidate tumor suppressor gene, *PTEN/MMAC1* was identified on chromosome 10q23.3, and frequent mutations in this gene were reported in GBMs as well as cancers of the breast, prostate, and kidney.[5, 6)]

In this study, we tried to elucidate the possible role of mutation of *PTEN/MMAC1* in carcinogenesis in various organs. We searched for genetic alterations in 178 tumors (11 GBMs, 14 astrocytomas, 47 breast cancers, 25 non-small cell lung cancers, 9 small cell lung cancers, 8 pancreatic cancers, 24 renal cell carcinomas, 20 ovarian cancers, and 20 metastatic lung tumors from tumors at various primary sites, including the breast, lung, kidney, urinary bladder, prostate, colon, and gall bladder) removed from Japanese patients at Tohoku University Hospital (Sendai) and its associated hospitals (Sendai). Clinical characteristics of these tumors are summarized in Table I. Samples were frozen in liquid nitrogen immediately after surgical resection and stored at $-80°C$ until use. DNAs were extracted according to methods described previously.[7)] In each case, the constitutional DNA was also prepared from either peripheral blood cells or from normal tissue.

Tumor DNAs were subjected to PCR-SSCP analysis to search for mutations. Primer sets for amplification of 9 exons of *PTEN/MMAC1* were designed according to Steck *et al.*,[6)] with some differences (see Table II). We divided exons 5 and 8 into two portions, because these two exons are relatively large. PCR-SSCP analysis was performed according to Orita *et al.*[8)] with some modifications.[9)] The PCR mixture was prepared as follows: 50 ng of genomic DNA, 2.5 pmol of [γ-^{32}P] end-labeled primer pair, 0.75 pmol each of deoxyribonucleotide triphosphates, 4.5 m*M* Tris-HCl (pH 8.8), 67 m*M* $(NH_4)_2SO_4$, 6.7 m*M* β-mercaptoethanol, 4.5 μM EDTA, 4.5 m*M* $MgCl_2$, and 0.25 unit of *Taq* DNA polymerase in a final volume of 10 μl. In each case, the amplified product was diluted two-fold with a formamide-dye mixture (95%

# The first two authors contributed equally to this work.

5 To whom correspondence should be addressed.

Abbreviations: GBM, glioblastoma multiforme; MMAC1, mutated in multiple advanced cancers 1; PTEN, phosphatase and tensin homolog deleted on chromosome ten; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism; LOH, loss of heterozygosity.

Table I. Clinical Characteristics of Samples

Origin of tumor	No. of tumors analyzed	No. of advanced tumors
Brain	25	11
Breast	47	10
Lung	34	20
Kidney	24	15
Ovary	20	12
Pancreas	8	6

formamide/0.25% bromophenol blue/0.25% xylene cyanol) and electrophoresed on a 5% polyacrylamide gel containing 5% glycerol at 4°C.

Typical examples of the SSCP analyses are shown in Fig. 1A. Extra-large migrating bands were observed in tumor GB8T (a case of GBM), whereas no alterations were found in the constitutional DNA of this patient (GB8N). These results suggested a somatic mutation in this tumor. Subsequently, the nucleotide sequences of the

Table II. Primers Used for Mutation Analysis of *PTEN/MMAC1*

Exon	Primer	Sequences (5′–3′)		Annealing temperature (°C)
		Forward	Reverse	
1	1F/1R	CAGCCGTTCGGAGGATTA	ATATGACCTAGCAACCTGACCA	55
2	2F/2R	TGACCACCTTTTATTACTCC	TACGGTAAGCCAAAAAATGA	55
3	3F/3R	ATATTCTCTGAAAAGCTCTGG	TTAATCGGTTTAGGAATACAA	55
4	4F/4R	TTCAGGCAATGTTTGTTA	CTTTATGCAATACTTTTTCCTA	55
5	5F/5IR	AGTTTGTATGCAACATTTCTAA	TTCCAGCTTTACAGTGAATTG	58
	5IF/5R	GACCAATGGCTAAGTGAAGAT	AGCAACTATCTTTAAAACCTGT	58
6	6F/6R	ATATGTTCTTAAATGGCTACG	CTTTAGCCCAATGAGTTGA	55
7	7F/7R	ACAGAATCCATATTTCGTGTA	TAATGTCTCACCAATGCCA	55
8	8F/8IR	TGCAAATGTTTAACATAGGTGA	GTAAGTACTAGATATTCCTTGTC	58
	8IF/8R	AGTCTATGTGATCAAGAAATCGA	CGTAAACACTGCTTCGAAATA	58
9	9F2/9R2	AAGATGAGTCATATTTGTGGGT	GACACAATGTCCTATTGCCAT	58

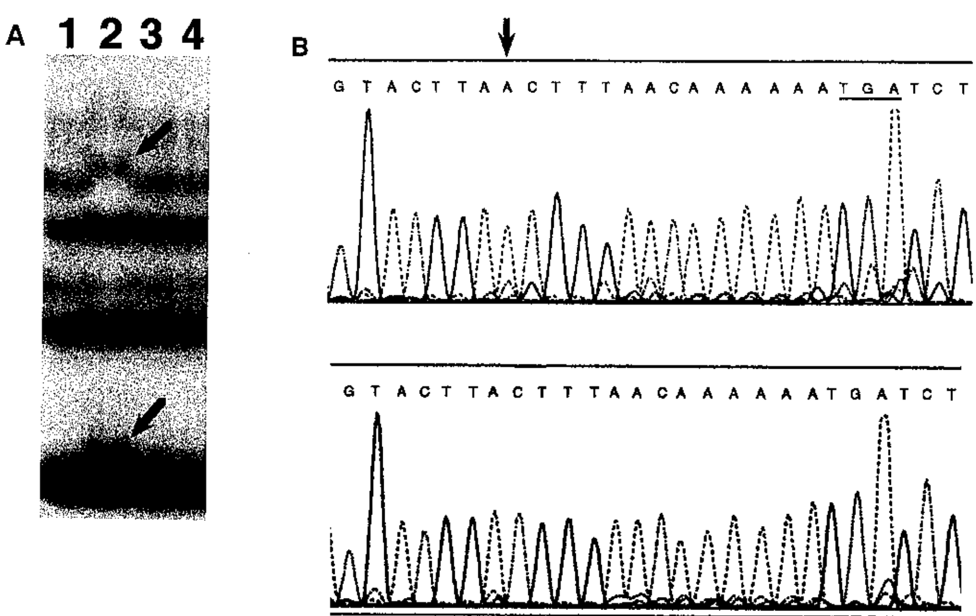

Fig. 1. Mutation analysis of the *PTEN/MMAC1* gene. A, Results of SSCP analysis. Lane 1, GB8N; lane 2, GB8T; lane 3, GB9T; lane 4, GB10T. Extra-large migrating bands, indicated by arrows, were observed in lane 2 (GB8T, a tumor with GBM), whereas no alterations were found in the corresponding normal tissue (in lane 1, GB8N). B, Nucleotide sequencing analysis of GB8T revealed a 1-bp insertion of A at codon 319 (indicated by an arrow in the upper row). The DNA sequence of the normal allele of this patient is shown in the lower row.

Table III. Results of PCR-SSCP Screening

	Mutations detected	Location	Type of alteration
Case GB8 (GBM)	ACT→AACT	Exon 8 319	Frameshift
Polymorphism	5 bp Insertion/deletion	Intron 4	Ins : del=53% : 47%

extra-large migrating bands were analyzed. We used a Thermo Sequenase dye terminator cycle sequencing premix kit (Amersham, Little Chalfont, UK) and an "ABI PRISM" 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA).[10, 11] The results are shown in Fig. 1B: a frameshift mutation due to one base insertion of A at codon 319 (ACT to AACT) was found. Nucleotide sequences of both strands were determined to confirm the results. This alteration generates a termination codon at 18- to 20-bp downstream (see Fig. 1B). Other than this mutation, we only found one polymorphism: a 5 bp insertion/deletion polymorphism at 106- to 110-bp downstream from the 5′ end of intron 4. The results of our mutation search are summarized in Table III.

We further analyzed LOHs in GBMs and astrocytomas utilizing the insertion/deletion polymorphism in the *PTEN/MMAC1* gene. Primers used were 4IF (5′-GAGTCATCCAGATTATCGAGA-3′) and 4R (listed in Table II), and the sizes of the products were 108/103 bp. Microsatellite markers at or near the *PTEN/MMAC1* locus, D10S215, AFMa086wg9, and D10S541, were also used. Although incidences of heterozygosity of these markers were not high in our samples, four (50%) of eight GBMs showed LOHs at or near the *PTEN/MMAC1* locus, whereas only one (17%) of six astrocytomas showed LOH (data not shown). Tumor GB8T had a two-hit mutation: LOH was found in addition to the somatic frameshift mutation of *PTEN/MMAC1* in this tumor.

Frequent mutations of the *PTEN/MMAC1* gene as well as homozygous deletions have been reported in cell lines and xenografts of various tumors.[5, 6] Although the number of tumors analyzed was not large, mutations in this gene in primary tumors were also reported with the frequencies of 16–17% in gliomas, 14% in breast cancers, and 17% in kidney cancers.[5, 6] In the present study, we detected only one (9%) mutation in 11 GBMs and no mutations were detected in other tumors in our series of Japanese cancer patients. There are several possible explanations of this observation: (1) there is a limitation of sensitivity in using the PCR-SSCP method to detect mutations in the *PTEN/MMAC1* gene, (2) mutations of the *PTEN/MMAC1* gene do not play a major role in the cancer types we analyzed in Japanese patients, or (3) mutations in the *PTEN/MMAC1* gene are more frequent in cell lines and/or xenografted tumors than the primary tumors. Since SSCP is a method with high sensitivity and is used for detection of mutations in many genes, we think that possibility 2 is more likely than possibility 1. Since we did not examine cell lines or xenografted tumors derived from Japanese patients, we cannot exclude possibility 3. Further studies are necessary to understand the role of the *PTEN/MMAC1* gene in human carcinogenesis.

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