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Mutational analysis of the PTEN gene localized at chromosome 10q23 in Thai patients with gliomas

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ABSTRACT Phosphatase and tensin homologue deleted on chromosome 10 (*PTEN*), a tumor suppressor gene located on chromosome 10q23, has recently been shown to act as a phosphatidylinositol 3,4,5-triphosphate phosphatase and to modulate cell growth and apoptosis. Genetic alterations in the *PTEN* tumor suppressor gene occur in several types of human cancers including glioblastoma, prostate, and breast. We examined genetic alterations of the *PTEN* gene in human gliomas. We screened 28 astrocytic tumors for mutations throughout the *PTEN* coding regions using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP), Klenow treatment and direct DNA sequencing of aberrantly migrating PCR products. We found nucleotide changes in introns but no mutations that caused amino acid alterations. Interestingly, the intronic mutations accompanying silent mutations were found only in patients with glioblastomas. In conclusion, the majority of astrocytic tumors do not carry mutations of the *PTEN* gene, which suggests that there may be other genes on chromosome 10 which are important in the tumorigenesis of gliomas.

KEYWORDS: *PTEN* gene, Gliomas, PCR-SSCP, Klenow treatment.

INTRODUCTION

Gliomas, in particular those of astrocytic origin, are the most common primary brain tumors and account for more than 40% of all central nervous system neoplasms.[1,2] In Thailand, Silivastanakul and coworkers[3] have reported that age-standardized rates for cancers of the nervous system are 1.8 per 100,000 in men and 1.4 per 100,000 in women. Astrocytomas are the most frequent neoplasms in Thai patients with brain tumors. These tumors are progressive, tend to recur following treatment, and are usually fatal. In spite of their impact on human health, the mechanisms involved in the pathogenesis of astrocytomas remain relatively unknown. Advances in molecular biology have shown that cancers progress as a result of several sequential genetic events involving either the activation of oncogenes or the inactivation of tumor suppressor genes.[4] Recent reports have indicated that a tumor suppressor gene located on 10q23 that is called *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) may account for the high incidence of chromosome 10 loss seen among astrocytic tumors .[5,6] The *PTEN* cDNA contains 9 exons and encodes a 403 amino acid peptide with a relative molecular mass of 47,000. Two major transcripts of approximately 2 and 5 kb and several other minor RNA species were detected in a wide variety of cell lines.[7,8] The protein encoded by this gene has domains homologous to protein phosphatases and the cytoskeletal proteins tensin and auxillin. The incidence of inactivating mutations in primary glioblastoma tumor samples is about 20%, whereas glioma cell lines exhibit a combined homozygous deletion and mutation rate approaching 60%.[9] The *PTEN* gene is also mutated in 80% of the autosomal dominant cancer predisposition disorder, Cowden disease and Bannayan-Zonana syndrome.[8] In human gliomas, it appears that *PTEN* mutations occur frequently in high-grade astrocytomas (WHO

grades III and IV).[10] In addition, loss of heterozygosity (LOH) from chromosome 10q23 is a hallmark of glioblastomas, the most malignant form of gliomas. A candidate tumor suppressor gene, *PTEN*, that may be targeted for deletion is associated with chromosome 10. To assess the role of the *PTEN* gene, we used PCR-SSCP analysis to detect its mutations. SSCP analysis is widely used to detect gene mutations mainly due to its simplicity and ability to screen a large number of samples quickly.[11] The present study was therefore designed to evaluate the incidence of *PTEN* mutations in 28 gliomas using PCR-SSCP analysis with additional Klenow treatment and DNA sequencing.

Materials and Methods

Tumor specimens and DNA extraction

Specimens of gliomas were obtained at operations for tumor removal or by biopsy from the Neurosurgery Sections (Siriraj and Ramathibodi Hospitals), Department of Surgery, Faculty of Medicine, Mahidol University, between 1995-2000. The specimens consisted of eight astrocytomas (WHO grade II), two gemistocytic astrocytomas (WHO grade II), three anaplastic astrocytomas (WHO grade III), and fifteen glioblastomas (WHO grade IV). Peripheral blood leukocytes from all patients were also prepared as a normal control. High molecular weight DNA was isolated from 28 frozen specimens and leukocytes by the method of Blin and Stafford.[12]

Polymerase Chain Reaction (PCR)

Oligonucleotide primers for amplification of all exons 1-9 as shown in Table 1, including exon-intron boundaries of the *PTEN* gene, were as previously reported by Sakurada et al.[13] These amplified DNA fragments covered all coding regions (from codon 1 to codon 403) of the *PTEN* gene. The PCR mixture, 25 µl in volume, contained 100 ng of genomic DNA, 2x thermophillic-magnesium free buffer (50 mM KCl, 10 µM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 % Triton X-100, 50 % glycerol (v/v)), 2.0 mM $MgCl_2$, 0.2 mM each of deoxyribonucleotide triphosphates, 1.0 µM of each primer and 0.5 Unit *Taq* DNA polymerase (Pharmacia). Thirty-five cycles of the reaction at 95 °C for 30 sec, at 55 °C for 1 min, 72 °C for 1 min in exons 1, 2, 3, 4, 5b, 6 and 7, or 95 °C for 30 sec, at 58 °C for 1 min, 72 °C for 1 min in exons 5, 8, 8b and 9 were performed with the Perkin-Elmer Cetus 9600 Thermal Cycler.

Klenow treatment

After completion of PCR, an equal volume (25 µl) of Klenow enzyme solution containing 0.2 unit/ml Klenow fragment of DNA polymerase I, 10 mM $MgCl_2$, 50 µM 4dNTP and 10 mM Tris-HCl (pH 8.3) was added and incubated at 37 °C for 15 min, as previously described by Kukita et al[14] and Thangnipon et al.[15] The additional of Klenow treatment after PCR amplification was an effective means to get rid of some extra bands on SSCP gel by the removal of protruding 3' tails from the PCR products.[15]

SSCP analysis

Two to six microliter aliquots of PCR product were added to 10 µl of formamide-dye solution. To denature double-strand PCR products, the mixture was heated at 95 °C for 10 to 15 min. Ten µl of each sample was loaded on a 5% non-denaturing polyacrylamide gel (0.035x40x30 cm) in 0.5x TBE (pH 8.3) with 5% glycerol. Electrophoresis was

Table 1. Amplified DNA fragments and primers used for the analysis of the *PTEN* gene mutations.

Exon	Primer	Sequences (5′ → 3′)		size (bp)
		Forward	Reverse	
1	1U/1L	CAG CCG TTC GGA GGA TTA	ATA TGA CCT AGC AAC CTG ACC A	484
2	2U/2L	TGA CCA CCT TTT ATT ACT CC	TAC GTT AAG CCA AAA AAT GA	370
3	3U/3L	ATA TTC TCT GAA AAG CTC TGG	TTA ATC GGT TTA GGA ATA CAA	444
4	4U/4L	TTC AGG CAA TGT TTG TTA	CTT TAT GCA ATA CTT TTT CCT A	315
5	5U/5L	AGT TTG TAT GCA ACA TTT CTA A	TTC CAG CTT TAC AGT GAA TTG	221
	5bU/5bL	GAC CAA TGG CTA AGT GAA GAT	AGC AAC TAT CTT TAA AAC CTG T	572
6	6U/6L	ATA TGT TCT TAA ATG GCT ACG	CTT TAG CCC AAT GAG TTG A	426
7	7U/7L	ACA GAA TCC ATA TTT CGT GTA	TAA TGT CTC ACC AAT GCC A	428
8	8U/8L	TGC AAA TGT TTA ACA TAG GTG A	GTA AGT ACT AGA TAT TCC TTG TC	262
	8bU/8bL	AGT CTA TGT GAT CAA GAA ATC GA	CGT AAA CAC TGC TTC GAA ATA	283
9	9U/9L	AAG ATG AGT CAT ATT TGT GGG T	GAC ACA ATG TCC TAT TGC CAT	331

carried out at 25 °C, 40 W for 2-6 hrs, depending on the size of the DNA fragments, as previously reported by Thangnipon et al.[15] The gel was stained using the silver staining method described by Bassam et al[16] with slight modifications. Briefly, each gel was fixed for 10 min in 10% ethanol and for 5 min in 100 mM HNO_3 and then stained for 20 min in 12 mM $AgNO_3$. After staining, the gel was soaked in chilled developing solution (3% Na_2CO_3 and 37% formaldehyde) until the appropriate signal was visualized, then immersed in 10% acetic acid for 5 min to stop the reaction. The gel was then dried and photographed.

DNA sequencing

Tumor samples exhibiting SSCP mobility shift bands were analyzed for nucleotide sequences by automated DNA sequencer (ABI PRISM model 377 version 2.1.1) using a dideoxy terminator cycle sequencing kit (Applied Biosystems, USA). The sequencing primers were the same as those used for the PCR-SSCP.

Results and Discussion

We examined 28 gliomas for mutations of the *PTEN* gene using the PCR-SSCP analysis with additional Klenow treatment, which covers the entire 9 exons and the flanking intron regions. We detected aberrantly migrating PCR bands from exon 4 in 6 out of 28 cases of gliomas (5 glioblastomas, 1 grade II astrocytoma). The nucleotide sequences of the aberrantly migrating bands were compared with the reported sequences. Chromatograms of the DNA sequence from two glioblastomas (cases 2 and 15) are shown in Figure 1 and one of the grade II astrocytomas (case 26) is shown in Figure 2. In case 2 the nucleotide variations in the intronic sequence are at positions 65 (t→g), 64 (a→t), 63 (g→t), 60 (c→g), 58 (t→c) and 51(c→t) upstream to the 5′ end of exon 4. The nucleotide changes in intronic sequence in case 15 are at position 64 (a→t) upstream of the 5′ end of exon 4. In case 26, the nucleotide changes in intronic sequence variations at position 64 (a→t), 63 (g→a), 62 (t→g) and 61(a→ t) upstream to the 5′ end of exon 4. No specific mutation was detected in the coding regions. Our results suggest that mutations of the *PTEN* gene do not play a major role in carcinogenesis, at least in the tumor types analyzed. Intron inclusion may be favored if the size of the intron does not yield a redefined exon greater than 300 bp.[17] The *PTEN* gene consists of large introns (greater than 300 bp) and, thus, disruption of the 5′ splice site in the *PTEN* gene is unlikely to result in read through of the entire downstream intron. Interestingly, intronic alterations of the *PTEN* gene have been reported in various tumors such as ovarian cancer[18], gliomas[19] and Cowden syndrome.[8] Our results are similar to the study of *PTEN* mutations in glioblastomas by Somerville et al[20], who have reported that inactivating mutations have been missed as they occur in noncoding regions of *PTEN* such as the promoter or introns. Sakurada et al[13] found only one (9%) mutation in 11 glioblastomas. No mutations were detected in other tumors such as breast, lung, pancreas, kidney and ovary in their series of Japanese cancer patients. This indicates that mutations in *PTEN* occur at low frequency in malignant glial tumors.

Mutations outside the coding exons could have inactivated the *PTEN* gene product by altering gene expression or splicing. Most of the tumors in the present series were the subject of our previous studies involving LOH analysis and *p53* mutations .[21] We have found LOH on chromosome 10 in 2 of 10 (20%) of glioblastomas and 1 of 6 (16.7%) of grade II astrocytomas (our unpublished data), but none of these tumors showed mutations in exons 1-9 of the *PTEN* gene. This supports previous data on gliomas[22], involving LOH analyses and mutation screening sequence data. There were no *PTEN* mutations identified among these gliomas, and this suggests that 10q LOH and *PTEN* mutations may not be linked. About 75-95% of the glioblastomas exhibit LOH at *PTEN*, but only about 10-30% of the samples have been shown to have mutations affecting the gene.[23] An alternative explanation for the absence of *PTEN* gene mutations in these patients with LOH on chromosome 10 is the possibility of a second tumor suppressor gene on chromosome 10 distinct from *PTEN*.

In conclusion, the majority of astrocytic tumors did not carry mutations of the *PTEN* gene which suggests that there may be other genes on chromosome 10 which are important in the tumorigenesis of gliomas.

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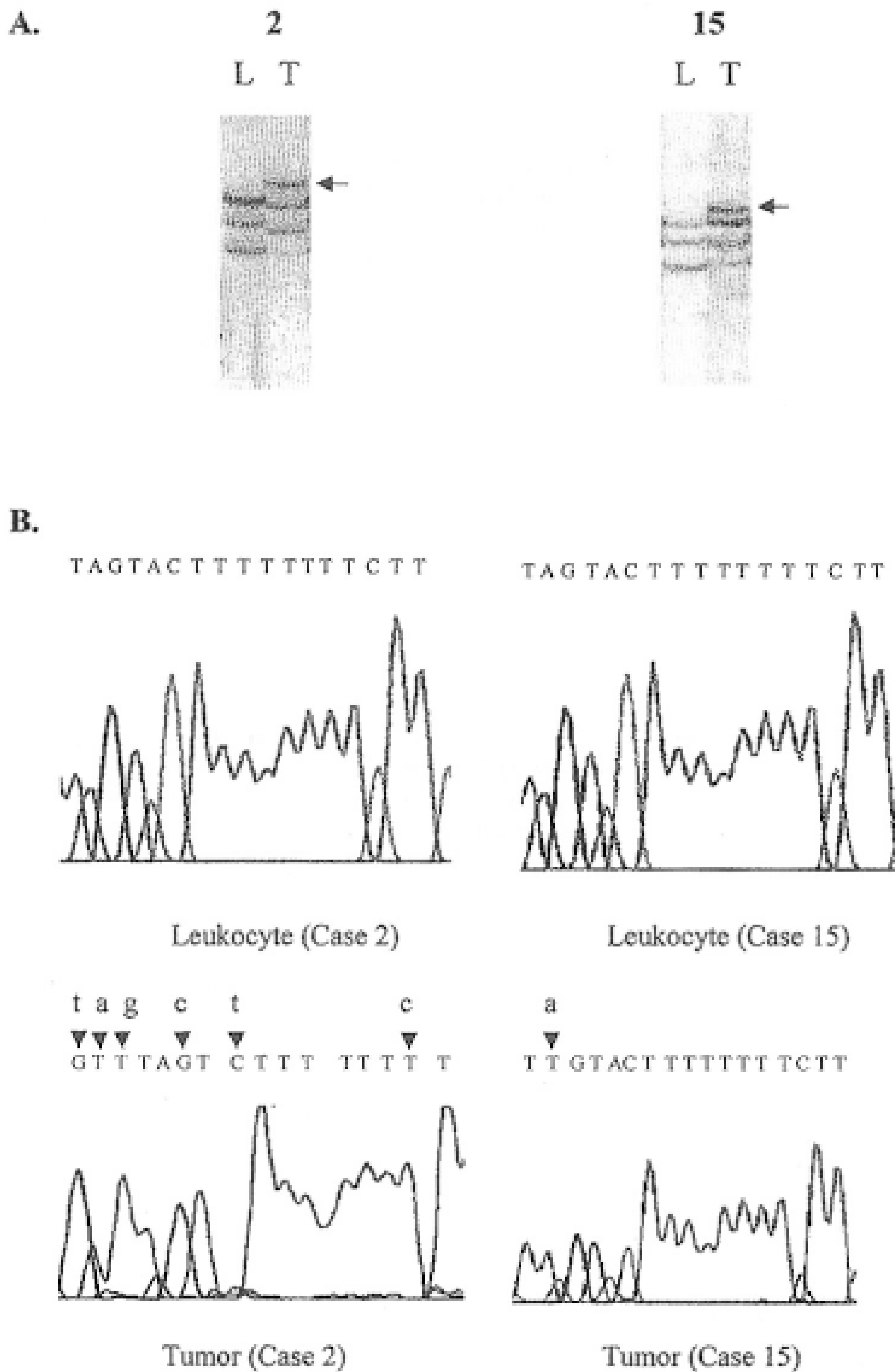
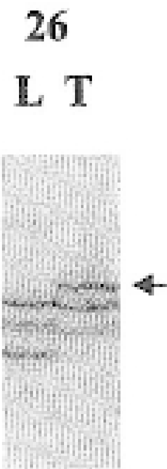


Fig 1. Analysis of the *PTEN* from glioblastomas (cases 2 and 15). A) PCR-SSCP analysis of exon 4 with Klenow treatment from leukocyte (L) and tumor (T) DNAs. The arrows indicate migration shifts. B) Automated DNA sequence analysis. (Upper) Normal sequence (cases 2 and 15); (Lower) The arrowheads indicate the nucleotide changes in intronic sequence variations at positions 65 (t $\rightarrow$ g), 64 (a $\rightarrow$ t), 63 (g $\rightarrow$ t), 60 (c $\rightarrow$ g), 58 (t $\rightarrow$ c) and 51 (c $\rightarrow$ t) before the 5′ end of exon 4 from case 2. The arrowheads indicate the nucleotide changes in intronic sequence variations at position 64 (a $\rightarrow$ t) before the 5′ end of exon 4 from case 15.



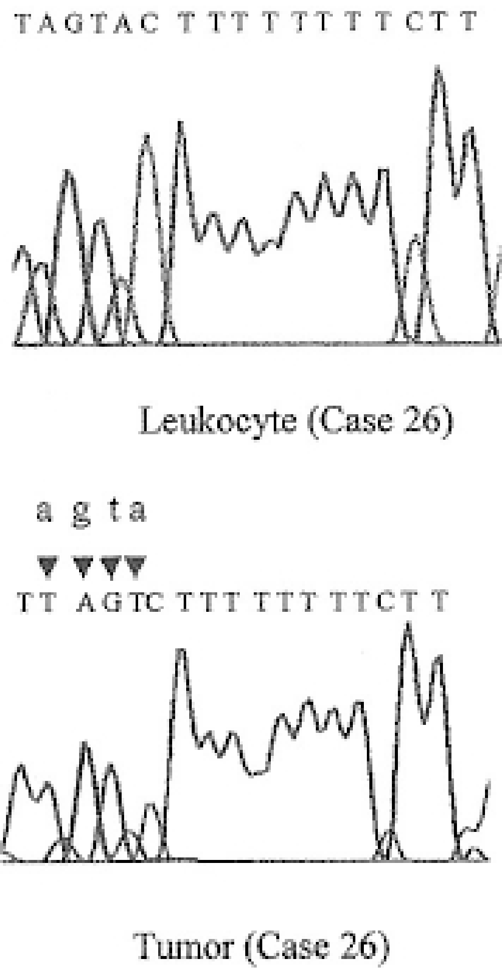


Fig 2. Analysis of the *PTEN* from grade II astrocytomas (case 26). A) PCR-SSCP analysis of exon 4 with Klenow treatment from leukocyte (L) and tumor (T) DNAs. The arrow indicates a migration shift. B) Automated DNA sequence analysis. (Upper) Normal sequence; (Lower) The arrowheads indicate the nucleotide changes in intronic sequence variations at positions 64 ($a \rightarrow t$), 63 ($g \rightarrow a$), 62 ($t \rightarrow g$) and 61($a \rightarrow t$) before the 5′ end of exon 4.

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