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Presence of Allelic Loss and PTEN Mutations in Malignant Gliomas from Malay Patients

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Summary

Loss of heterozygosity (LOH) on several loci and mutations on PTEN tumor suppressor gene (10q23.3) occur frequently in sporadic gliomas. We have performed polymerase chain reaction (PCR)-LOH analysis using microsatellite markers and single-stranded conformational polymorphism (SSCP) analysis to determine the incidence of allelic losses on chromosome 10q, 9p, 17p and 13q and mutations of exons 5, 6 and 8 of the PTEN gene in malignant gliomas. Twelve of 23 (52.2%) malignant glioma cases showed allelic losses whereas 7 of 23 (30.4%) samples showed aberrant band patterns and mutations of the PTEN gene. Four of these cases showed LOH on 10q23 and mutations of the PTEN gene. The data on LOH indicated the involvement of different genes in gliomagenesis whereas mutations of the PTEN gene indicated the role of PTEN tumor suppressor gene in the progression of glioma in Malay population.

Key Words: *Glioma, Loss of heterozygosity, Microsatellite markers, PTEN, Single-stranded conformational polymorphism*

Introduction

Increasing number of genetic alterations was found to involve in tumor progression. One of the alterations that occur at high frequency in a variety of human tumors is loss of heterozygosity (LOH) at chromosome 10q23. This change appears to occur late in tumor development. Although rarely seen in low-grade glial tumors, LOH at 10q23 occurs in approximately 70% of glioblastomas[1,2]. The pattern of LOH and the finding that wild-type chromosome 10 suppresses the tumorigenicity of glioblastoma cells in mice suggest that 10q23 encodes a tumor suppressor gene which involve in gliomagenesis[3].

Loss of heterozygosity at non-random frequency of different loci has been consistently reported in sporadic gliomas[1, 4-8]. The most documented regions of allelic losses include 9p21, 10q23-25 and 17p13[4]. Although not strongly associated with loss of heterozygosity in brain tumors, allelic losses on chromosome 13q in malignant astrocytomas have been documented previously[5]. Frequent LOH at 10q23 and mutations of the tumor suppressor gene located on 10q23.3, the PTEN (phosphatase and tensin homologue deleted from chromosome 10) gene have been found in various types of cancer, including gliomas[3,6]. The mutations were found to be distributed along the entire PTEN gene[6].

In malignant astrocytomas, consistent and frequent allelic losses were detected on chromosomes 10, 13q, 17p and 22q, suggesting the presence of tumor

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suppressor genes on these chromosomes[5]. In glioblastomas with partial loss of chromosome 10, at least three common deletions have been identified; 10p14-pter, 10q23-24 and 10q25-qter, suggesting the presence of multiple tumor suppressor genes[7]. In 40-60% of glioblastomas, deletion was found involving chromosome 9p21[8]. LOH on chromosome 17p was observed in anaplastic oligodendroglioma cases[5]. LOH on chromosome 10[9] and 13q[5] was found in most anaplastic ependymoma cases.

PTEN gene abnormalities have been identified in various types of human carcinoma, including gliomas [3,10-14]. Mutations of PTEN gene are restricted to high-grade rather than low-grade gliomas and may be associated with the transition from a low histological grade to anaplasia[1,15-17].

In gliomas, PTEN mutations are preferentially found in glioblastoma multiformes[1]. Deletions of the PTEN region at 10q23 are also reported as predominant findings in glioblastomas[1,3,18]. PTEN aberrations are detectable in a low fraction (<10%) of anaplastic astrocytomas and anaplastic oligodendrogliomas and when present, indicate a poor prognosis[19]. PTEN mutations are either rare or absent in WHO grade I and II astrocytic, oligodendroglial and mixed gliomas, glioneuronal tumors, as well as low-grade and anaplastic ependymal tumors[19].

The present research focused on detecting allelic losses or LOH on specific loci on chromosomes 10q, 9p, 17p and 13q which might harbors genes or tumor suppressor genes. In addition, mutational analysis was performed to identify the mutations occurring on exons 5, 6 and 8 of the PTEN gene, which is a tumor suppressor gene frequently implicated in sporadic brain tumors.

Materials and Methods

Specimens and DNA Samples

23 malignant glioma specimens from Malay patients referred to Universiti Sains Malaysia Hospital (HUSM), Kelantan were collected. The samples included 7 glioblastoma multiforme, 10 anaplastic astrocytoma, 4 anaplastic oligodendroglioma and 2 anaplastic ependymoma. Genomic DNA was extracted from the tumor tissues for LOH and PTEN mutational analysis. Normal samples were obtained from tissue samples that had been diagnosed histopathologically as having no tumor cells and from peripheral blood of persons with no major genetic disease.

PCR-LOH Analysis using Microsatellite Markers

LOH was detected in the DNA samples using highly polymorphic microsatellite markers on chromosome 10q, D10S532 (10q11), D10S541 (10q23) and D10S216 (10q25); chromosome 9p21, D9S165 (9p21) and D9S162; chromosome 17p13, D17S786 and D17S1176 and chromosome 13q12, D13S289 and D13S171 (http://www.gdb.org). Non-radioactive PCR method was performed in a reaction mixture of 50ml. 30 cycles of PCR were performed using Eppendorf Mastercycler (Eppendorf, GmbH Germany). PCR products were loaded onto a denaturing polyacrylamide gel and electrophoresed. The gel was then visualized by silver staining. All samples in which two distinct alleles of similar intensity which present in the normal DNA were considered to be informative. LOH was scored as positive when a clear reduction of signal intensity (or more than 50%) detected in one of the alleles of the tumor DNA compared with the paired normal DNA[20]. In instances where more than one band was present within each allele, the exact position of each allele was decided by comparing the banding pattern in all samples analyzed with the same marker and selecting the most consistent pattern: the two alleles were identified as two groups of bands of similar number and similar signal intensity. Densitometry was undertaken on samples where the reduction in signal intensity was difficult to quantify visually. All samples showing LOH were subjected to repeat amplification and analysis for confirmation.

PCR-SSCP Analysis of PTEN Gene

Prescreening for mutations on the PTEN gene was carried out by PCR-SSCP analysis of exons 5, 6 and 8 using previously described primers[12]. All PCR reactions were performed with a Eppendorf Gradient Mastercycler (Eppendorf, GmbH Germany) in a 50μl volume assays, including 100ng of genomic DNA, 25pmol of each oligonucleotide primer, 0.2mM of dNTP mix, 1X PCR Buffer containing Tris-HCl (pH 8.8), $(NH_4)2SO_4$ and 0.1% Tween 20, 0.05U/ml of Taq DNA Polymerase (Fermentas GmbH, Germany) and 2.0mM of $MgCl_2$, plus 6% dimethyl sulfoxide for the primer set of exon 5. After an initial denaturation at 94°C for 3 minutes, 30 cycles of denaturation (94°C for 1 minute), annealing (55°C for the primer set of exon 5 and 58°C for the primer set of exon 6 and 8) and extension (72°C for 1 minute) were performed. The final extension was

performed for 10 minutes. For SSCP analysis, 8µl of the PCR product were mixed with 4µl of sequencing gel loading dye (Amresco, USA) and were heat-denatured at 95°C for 4 minutes. The denatured products were then immediately cooled in ice. 10ml of samples of exon 6 were loaded onto an MDE gel (FMC Corp, Rockland, ME) containing 10% glycerol, while samples of exons 5 and 8 were analyzed using an 8% polyacrylamide gel containing 10% glycerol. To obtain optimal separation of the single-stranded conformers, the ratio of methylene-bis-acrylamide to acrylamide was 1:19. Gel electrophoresis was performed using DCode™ Universal Mutation Detection System gel apparatus (BioRad Laboratories Ltd, UK) at 10 to 15W for 16 hours. The temperature was maintained at 17°-22°C using the apparatus. The gel was then visualized by silver staining[21,22] after completion of electrophoresis.

DNA sequence analysis of PTEN gene
PCR products of the PTEN gene with variant SSCP patterns were purified using PCR Purification Kit (QIAgen GmbH, Germany) before proceeding with direct sequence analysis.

Results

LOH Analysis on 10q, 9p, 17p and 13q
12 of 23 (52.2%) glioma cases showed loss of heterozygosity (Figure 1). Of 12 cases with allelic losses, 7 (58.3%) cases demonstrated loss of heterozygosity on chromosome 10q, 3 (25.0%) cases showed allelic loss on chromosome 9p, 4 (33.3%) cases exhibited loss of heterozygosity on chromosome 17p and 2 (16.7%) cases were found to be positive for loss of heterozygosity on chromosome 13q. The cases include 5 (41.7%) cases of glioblastoma multiforme, 3 (25.0%) cases of anaplastic astrocytoma, 3 (25.0%) cases of anaplastic oligodendroglioma and 1 (8.3%) case of anaplastic ependymoma. 4 cases showed loss of heterozygosity on more than one locus. Distribution of loss of heterozygosity cases on chromosomes 10q, 9p, 17p and 13q are summarized in Table I.

Mutational Analysis of PTEN Gene
PCR-SSCP analysis of the PTEN gene showed aberrant band patterns in 7 out of 23 (30.4%) samples (Figure 5-7) involving exons 5 and 6 of the PTEN gene. Out of 7 glioma cases with SSCP band alterations, 2 (28.6%) cases were glioblastoma multiforme, 4 (57.1%) cases were anaplastic astrocytoma and 1 (14.3%) case was anaplastic ependymoma. DNA sequence analysis of the samples with band alterations revealed mutations leading to amino acid changes of the PTEN gene. All aberrations detected were point mutations. Among the base substitutions, 71% were transitions (G:C → A:T, 5 cases) and 29% were transversions (G:C → C:G, 2 cases). 4 (57.1%) out of 7 samples revealed missense mutations and 3 (42.9%) out of 7 samples revealed nonsense mutations.

The cases with mutations on exon 5 include 2 anaplastic astrocytoma and 2 glioblastoma multiforme cases. Nonsense mutations were detected on codon 130 in both anaplastic astrocytoma cases whereas in the glioblastoma cases, missense mutations were detected on codon 105 and 124, resulting in the substitution of cystein for serine. Mutations of exon 6 were detected on 2 anaplastic astrocytoma, 1 anaplastic ependymoma and 2 glioblastoma multiforme cases. The distribution of missense mutations targeted on codon 173 in anaplastic astrocytoma and glioblastoma cases, resulting in the substitution of arginine by histidine in anaplastic astrocytoma and arginine by cysteine in glioblastoma. The mutation found in anaplastic ependymoma (codon 171) resulted in the introduction of premature stop codon. Among the cases with mutations, 2 glioblastoma multiforme cases showed aberrant band patterns and mutations on both exons 5 and 6, involving codons 105 and 124 of exon 5 and codon 173 of exon 6. SSCP band alterations and mutations were not detected in exon 8.

LOH on 10q and Mutations of PTEN Gene
Out of 7 informative cases of loss of heterozygosity on chromosome 10q and 7 cases of PTEN mutations, 4 cases showed mutations on PTEN gene accompanied by loss of heterozygosity on 10q23.

Table I: Distribution of loss of heterozygosity cases on chromosomes 10q, 9p, 17p and 13q in malignant gliomas according to age and gender.

Microsatellite markers	Primer Sequences (5′-3′) (http://www.gdb.org/)		Locus on Chromosome (http://www.gdb.org/)	Size of PCR amplicons (bp)
	Forward	Reverse		
D10S532	TGGTCTCTAGAAAAATTAATGCAAT	AAGTTGTTTGTGGGGAGTCA	10q22.3	267
D10S541	AAGCAAGTGAAGTCTTAGAACCACC	CCACAAGTAACAGAAAGCCTGTCTC	10q23.3	272
D10S216	TGGCAGAGCCATTTAACTAC	AGCTGCTGGGAATAATATGC	10q25.1	210
D9S165	GACTTTGGCTGCTAGATGTG	CAGAGGAGTTACAAATATAGACAGG	9p21	214
D9S162	GCAATGACCAGTTAAGGTTC	AATTCCCACAACAAATCTCC	9p21	186
D17S786	TACAGGGATAGGTAGCCGAG	GGATTTGGGCTCTTTTGTAA	17p13.3	149
D17S1176	ACTTCATATACATATCACGTGC	TCAATGGAGAATTACGATAGTG	17p13.1	95
D13S171	CCTACCATTGACACTCTCAG	TAGGGCCATCCATTCT	13q12.3	231
D13S289	CTGGTTGAGCGGCATT	TGCAGCCTGGATGACA	13q12.1	260

Table II: Mutations of the PTEN gene. 35% (7 of 20) showed band alterations in the SSCP analysis of PTEN gene. Mutations were only detected in exons 5 and 6. All aberrations were point mutations, including 4 (57%) missense mutations and 3 (43%) nonsense mutations. No frameshift mutations were involved. Among the base substitutions, 71% were transitions (G:C ‡ A:T, 5 cases) and 29% were transversions (G:C → C:G, 2 cases).

Primers	Primer sequence (5′Æ3′) (Sakai et al., 1998)		Exon size (bp)	Size PCR amplicons (bp)
	Forward	Reverse		
Exon 5	ACCTGTTAAGTTTGTATGCAAC	TCCAGGAAGAGGAAAGGAAA	239	379
Exon 6	CATAGCAATTTAGTGAAATAACT	GATATGGTTAAGAAAACTGTTC	142	274
Exon 8	CTCAGATTGCCTTATAATAGTC	TCATGTTACTGCTACGTAAAC	225	557

Table III: Distribution of loss of heterozygosity cases on chromosomes 10q, 9p, 17p and 13q in low- and high-grade gliomas according to age, gender and microsatellite markers.

Patient No. (Sex/Age, y)	Diagnosis (WHO Grade)*	LOH on 10q			LOH on 9p		LOH on 17p		LOH on 13q	
		D10S532	D10S541	D10S216	D9S165	D9S162	D17S786	D17S1176	D13S289	D13S171
22/97 (M, 32)	AA (III)	LOH	LOH	-	-	-	-	-	-	-
28/97 (F, 56)	AA (III)	-	-	-	-	-	-	-	-	-
26/98 (F, 41)	AA (III)	-	-	-	-	-	-	-	-	-
36/98 (M, 58)	AA (III)	-	-	-	-	-	-	-	-	-
40/98 (M, 36)	AA (III)	-	-	-	-	-	-	-	-	-
42/98 (M, 34)	AA (III)	-	LOH	-	LOH	-	-	-	-	-
9/99 (F, 46)	AA (III)	-	-	-	-	-	-	-	-	-
16/00 (F, 17)	AA (III)	-	-	-	-	-	-	-	-	-
19/00 (M, 12)	AA (III)	-	-	-	-	-	-	-	-	-
19/98 (M, 10)	PXA (III)	LOH	-	LOH	-	-	LOH	-	-	-
1/98 (M, 50)	AO (III)	-	-	-	-	LOH	LOH	-	-	-
8/98 (M, 48)	AO (III)	-	-	-	-	-	-	-	-	LOH
28/98 (F, 18)	AO (III)	-	-	-	-	-	-	-	-	-
4/99 (F, 49)	AO (III)	-	-	-	-	LOH	-	-	-	LOH
12/98 (F, 3)	AE (III)	-	-	-	-	-	-	-	-	-
2/99 (F, 3)	AE (III)	-	-	-	-	-	-	-	-	-
19/97 (M, 55)	GBM (IV)	-	-	LOH	-	-	-	-	-	-
20/97 (M, 8)	GBM (IV)	-	LOH	-	-	-	LOH	-	-	-
20/98 (F, 44)	GBM (IV)	-	-	-	-	-	-	LOH	-	-
27/98 (M, 36)	GBM (IV)	-	-	-	-	-	-	-	-	-
5/99 (M, 27)	GBM (IV)	-	LOH	-	-	-	-	-	-	-
16/99 (M, 40)	GBM (IV)	-	LOH	-	-	-	-	-	-	-
36/01 (F, 46)	GBM (IV)	-	-	-	-	-	-	-	-	-
No. of LOH/total samples (%)		7/23 (30.4%)			3/23 (13.0%)		4/23 (17.4%)		2/23 (8.7%)	
No. of LOH/total cases with LOH (%)		7/12 (58.3%)			3/12 (25.0%)		4/12 (33.3%)		2/12 (16.7%)	

*Diagnosis: AA = anaplastic astrocytoma, PXA = anaplastic pleomorphic xanthoastrocytoma, AO = anaplastic oligodendroglioma, AE = anaplastic ependymoma, GBM = glioblastoma multiforme

LOH = case with loss of heterozygosity, - = case which retain heterozygosity

Table IV: Mutations of the PTEN gene. 7 of 40 (17.5%) glioma cases showed band alterations in the SSCP analysis of PTEN gene. Mutations were detected in exons 5 and 6 and none were detection 8. All aberrations were point mutations, including 4 (57%) missense mulations and 3 (43%) nonsense mutations. Among the base aubstitutions, 71% were transitions (G:C → A:T, 5 cases) and 20% were transversions (G:C → C:G, 2 cases)

Patient No. (Sex/Age, year)	Diagnosis* (WHO Grade)	Exon with SSCP aberration	Codon	Features	Nucleotide change	Amino acid change
22/97 (M, 32)	AA (III)	NA				
28/97 (F, 56)	AA (III)	NA				
26/98 (F, 41)	AA (III)	NA				
36/98 (M, 58)	AA (III)	Exon 6	173	CpG; catalytic domain	CGC→CAC	Arg→His
40/98 (M, 36)	AA (III)	NA				
42/98 (M, 34)	AA (III)	Exon 6	173	CpG; catalytic domain	CGC→CAC	Arg→His
19/00 (M, 34)	AA (III)	NA				
9/99 (F, 46)	AA (III)	NA				
16/00 (M, 26)	AA (III)	Exon 5	130	Catalytic core motif; CpG	CGA→TGA	Arg→stop
19/98 (M, 10)	PXA (III)	Exon 5	130	Catalytic core motif; CpG	CGA→TGA	Arg→stop
1/98 (M, 50)	AO (III)	NA				
8/98 (M, 48)	AO (III)	NA				
28/98 (F, 18)	AO (III)	NA				
4/99 (F, 49)	AO (III)	NA				
12/98 (F, 3)	AE (III)	Exon 6	171	Catalytic domain	CAG→TAG	Gln→stop
2/99 (F, 3)	AE (III)	NA				
19/97 (M, 55)	GBM (IV)	NA				
20/97 (M, 8)	GBM (IV)	Exon 5	105	Catalytic domain	TGT→TCT	Cys→Ser
		Exon 6	173	CpG; catalytic domain	CGC→TGC	Arg→Cys
20/98 (F, 44)	GBM (IV)	NA				
27/98 (M, 36)	GBM (IV)	NA				
5/99 (M, 27)	GBM (IV)	Exon 5	124	Catalytic core motif	TGT→TCT	Cys→Ser
		Exon 6	173	CpG; catalytic domain	CGC→TGC	Arg→Cys
16/99 (M, 40)	GBM (IV)	NA				
36/01 (F, 46)	GBM (IV)	NA				

*Diagnosis: AA = anaplastic astrocytoma, PXA = anaplastic pleomorphic xanthoastrocytoma, AO = anaplastic oligodendroglioma, AE = anaplastic ependymoma, GBM = glioblastoma multiforme

M = male, F = female, NA = no abnormalities

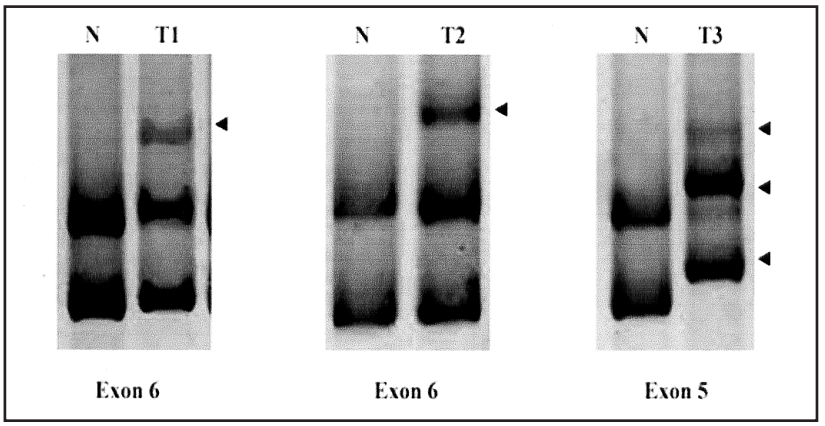

Fig. 1: Representative results of loss of heterozygosity (LOH) on chromosome 10q, 9p and 17p detected by using the microsatellite markers D10S541, D9S165 and D17S786, respectively in tumor samples from 3 patients with glioma. The arrow indicates LOH in tumor (T) DNA, with a 50%-90% reduction in signal intensity compared to corresponding normal (N) control. 16/99 represents glioblastoma multiforme and 42/98 and 19/98 represent anaplastic astrocytoma.

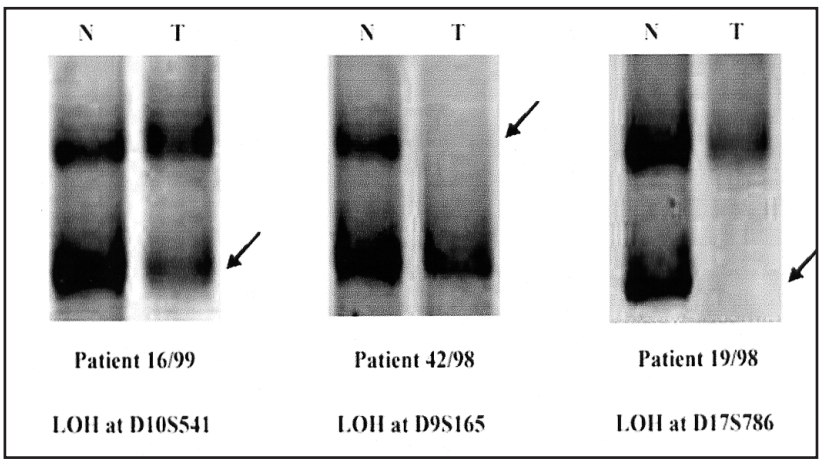

Fig. 2: Representative PCR-SSCP analysis of exons 5 and 6 of the PTEN gene. The arrows indicate SSCP band aberrations or shifts in tumour DNA (T), compared with corresponding normal (N) alleles. T1 represents glioblastoma multiforme and T2 and T3 represent anaplastic astrocytoma.

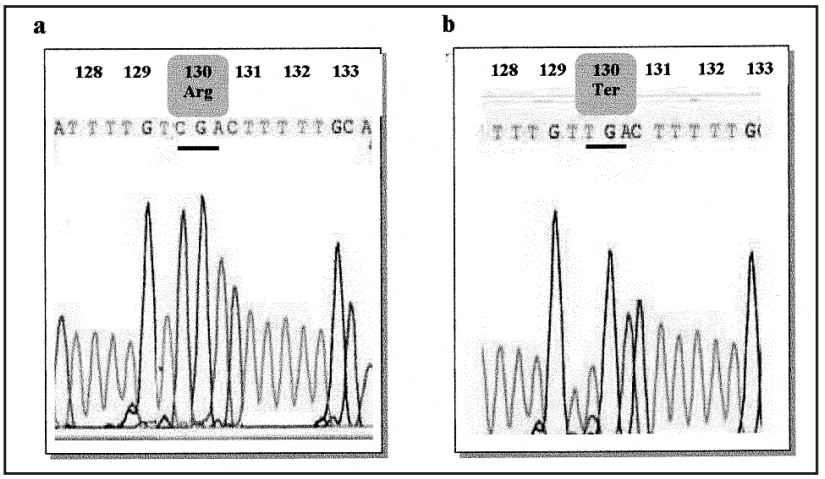

Fig. 3: Partial sequence chromatography of the PCR-amplified product of exon 5 of the PTEN gene. The glioblastoma sample analyzed revealed a missense mutation at codon 105, resulting in the substitution of cystein for serine.

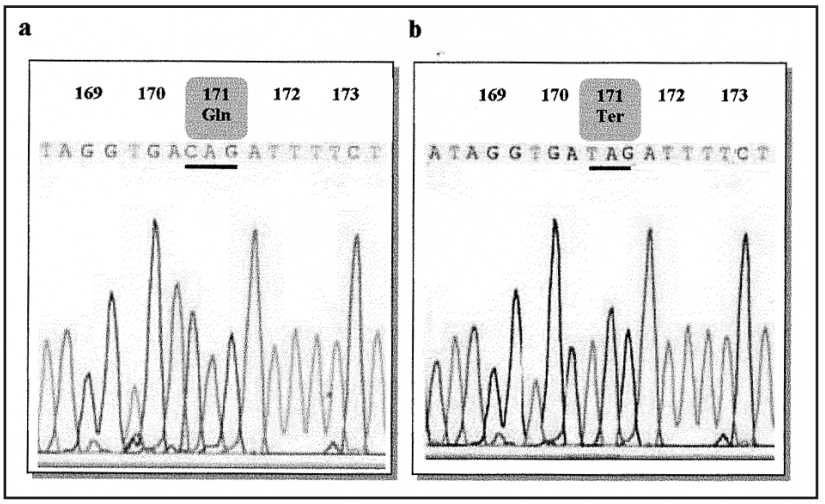

Fig. 4(a): Partial sequence electropherogram of exon 6 of the wild type *PTEN* gene.
(b): Partial sequence electropherogram of mutated exon 6 of the *PTEN* gene. The anaplastic ependymoma sample analyzed revealed a transition of CT at codon 171, resulting in a termination codon (CAG → TAG; Gln → Ter).

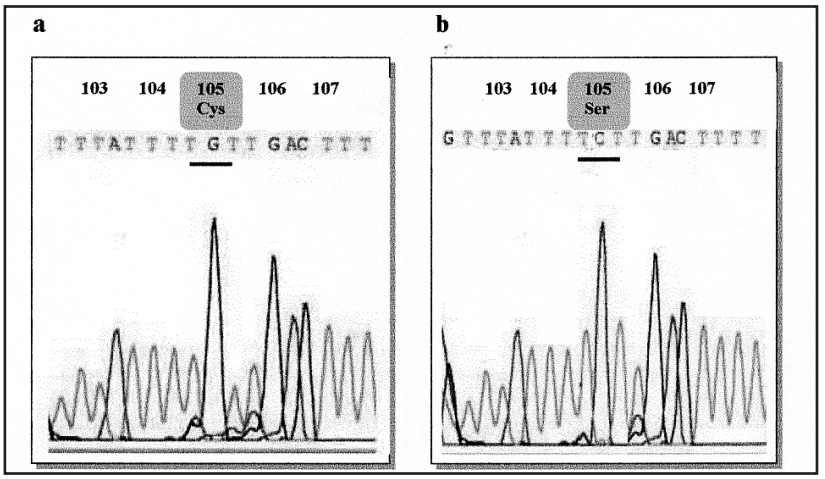

Fig. 5(a): Partial sequence electropherogram of exon 5 of the wild type *PTEN* gene.
(b): Partial sequence electropherogram of mutated exon 5 of the *PTEN* gene. The glioblastoma multiforme sample analyzed revealed a transversion of G → C at codon 105, resulting in the substitution of cysteine to serine (TGT → TCT; Cys → Ser).

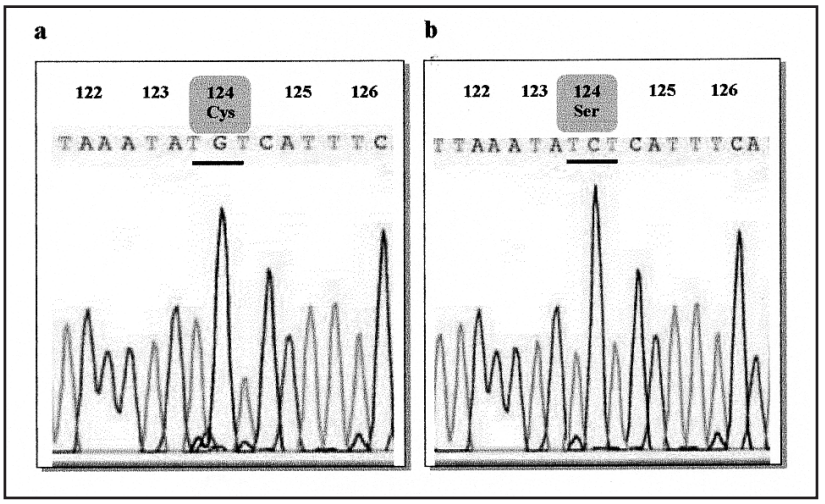

Fig. 6(a): Partial sequence electropherogram of exon 5 of the wild type *PTEN* gene.
(b): Partial sequence electropherogram of mutated exon 5 of the *PTEN* gene. The glioblastoma multiforme sample analyzed revealed a transversion of G → C at codon 124, resulting in the substitution of cysteine to serine (TGT → TCT; Cys → Ser).

Discussion

The present study is the first to demonstrate that loss of heterozygosity on chromosomes 10q, 9p, 17p and 13q and mutations of the PTEN gene involved in the progression of glioma in Malay population. The observed pattern of allelic losses demonstrated that loss of heterozygosity involved several loci and that the particular analyzed regions contain specific tumor suppressor genes, which might be targets for cancer-causing mutations or inactivation[23].

The highest number of cases with LOH were detected on chromosome 10q23.3 harboring the PTEN gene. This region was reported to be frequently deleted in LOH studies involving the q arm of chromosome 10 and fine mapping studies of the entire chromosome[6-8,18]. LOH was also detected on the regions 10q25.1, 10q22, 9p21 and 13q12.3. These regions have been documented to contain genes or tumor suppressor genes involved in several carcinoma, including malignant gliomas[4,5,8,24,25]. Less frequent LOH was detected on chromosomes 17p13.1 whereas no allelic loss was detected on the region 13q12.1, suggesting that these regions are not typically involved in the progression of malignant gliomas in Malay patients.

Eleven glioma cases retained their heterozygosity in this study. This presumably occurred because of loss or deletion of both alleles in the tumor DNA so that only the contaminating normal DNA was amplified to produce 2 alleles with low signal intensity. However, this can only be confirmed by performing a homozygous deletion analysis[26]. Interestingly, 2 of the tumors with retention of allele heterozygosity were glioblastoma multiforme, suggesting a relationship between malignant tumor pathology and more extensive deletions.

Most of PTEN gene mutations detected in the present study were anaplastic astrocytoma and glioblastoma. Five codons containing CpG dinucleotides were found mutated on exons 5 and 6 of the PTEN gene, which involved entirely missense and nonsense mutations. The codon most frequently affected or mutated was codon 173 which is conserved in tensin, auxilin and bacterial phosphatase[27]. The mutations of this gene which lead to amino acid substitutions may generally affect conserved residues or structurally conserved features of the protein, as demonstrated by Schmidt *et al.*[6]. It is believed that the N-terminal half of PTEN is functionally more significant for tumor suppression because of homology to tensin, auxilin and phosphatase, regions that may control cell cycle, invasion and metastasis[28]. These findings suggest that mutations of PTEN are concentrated to the N-terminal phosphatase domain with cluster of mutations in the region 5' to the core phosphatase motif and the 5'-end of exon 6.

The tumors containing PTEN mutations also showed loss of heterozygosity in the chromosome 10q23 region flanking the PTEN gene. The pattern of allelic losses on 10q23 as well as mutations of the gene itself appear to be associated with the progression of glioma[26,29] and indicated complete loss of the wild-type PTEN gene[30]. These findings also suggest that PTEN gene might be inactivated by point mutations or small deletions[26] and that both alleles of the PTEN gene were inactivated by a classical two-hit mechanism[31], therefore confirming the previous idea that PTEN acts as a tumor suppressor gene. However, the lack of detectable PTEN alterations in a considerable fraction of malignant gliomas with 10q loss suggests that at least one additional tumor suppressor gene responsible in the progression of glioma is located on 10q.

Thus, the data obtained on loss of heterozygosity analysis and mutational analysis of the PTEN gene provide relevant information on the presence of putative tumor suppressor genes that might be involved in the pathway of glioma progression in Malay population. In addition, these data provide useful evidence of molecular genetic alterations of malignant glioma in South East Asian patients, particularly in the East Coast of Malaysia.

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