ORIGINAL ARTICLE

Mutations of the *p53* Gene in Gliomas From Malay Patients

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Summary

This is the first investigation performed to detect the presence of the *p53* mutation in Malay patients with gliomas. The *p53* gene was amplified using polymerase chain reaction (PCR) from 33 fresh-frozen tumour tissues from patients histologically confirmed as glioma. Four hot spot areas that lie between exon 5 to 8 were screened for mutation by mean of non-isotopic "cold" single strand conformation polymorphism (SSCP) analysis and direct sequencing. The frequency of *p53* gene mutation in gliomas examined was 33% (11 of 33). Five (45.5%) cases had mutation in exon 7, four (36.4%) had mutation in exon 8 and two (18.1%) had mutation in exon 6. Seven (63.6%) of 11 mutations were single nucleotide point mutations of which 5 were missense mutations, 1 was nonsense mutation and 1 was silent mutation. Three (27.3%) showed insertion mutation and 1 (9.1%) showed deletion mutation. Of the point mutations, 57.1% were transitions and 42.9% were transversions. These results suggested that *p53* mutations frequently occur in gliomas and this gene does play an important role in the tumourigenesis process of Malay patients with brain tumours.

Key Words: Gliomas, *p53* gene mutation, Malay

Introduction

Among the tumours of the central nervous system, gliomas are the most common[1]. Gliomas are neuro-epithelial tumours arising from supporting glial tissue and are classified morphologically as astrocytomas, oligodendrogliomas, ependymomas and mixed tumours. Astrocytomas, the most common category, include a spectrum of tumours ranging from slow-growing juvenile pilocytic astrocytomas to highly malignant glioblastoma multiforme[2,3].

Genetic changes are known to characterize the genesis of human tumours, and the involved genes are typically oncogenes and tumour suppressor gene[4]. One of the known molecular alterations in gliomas is the inactivation of the tumour suppressor gene *p53*. The human *p53* gene is located in the 20 kb of chromosome band 17p13.1 and consists of 11 exons[5,6]. The majority of mutations in *p53* gene occur in 4 hot spot regions within exon 5 to 8, which represent evolutionarily highly conserved sequences[6,7,8]. Genetic alterations of the *p53* gene such as point mutation, deletion or

This article was accepted: 20 November 2002

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insertion, often result in mutant *p53* protein which have an altered conformational structure. Missense mutations in the *p53* gene are the most common[5,9].

p53 gene inactivation represents the genetic event most frequently involved in the tumorigenesis process of astrocytic gliomas[4]. Most studies of *p53* gene in gliomas used the polymerase chain reaction (PCR) and single strand conformation polymorphism (SSCP) analysis to detect mutations[8,10-14]. Previous studies showed that *p53* mutations occurred with an overall frequency of 26% in grade II, 25% in grade III and 39% in grade IV astrocytomas[15]. The aim of this study was to investigate whether genetic alterations of *p53* gene is involved in the development of gliomas in Malay patients.

Materials and Methods

Tumour specimens and DNA extraction

Thirty-three specimens of gliomas were studied from the Brain Tumour Tissue Bank, of the Neuroscience Unit, School of Medical Sciences, Universiti Sains Malaysia. These gliomas were taken from patients between 1996 till early 2000 after ethical committee and patients' approval. The tumours were classified according to World Health Organization (WHO) brain tumour scheme[2,3]. They were classified as 8 juvenile pilocytic astrocytoma (Grade I), 3 astrocytoma (Grade II), 1 pleomorphic xantoastrocytoma (Grade II), 9 anaplastic astrocytoma (Grade III), 6 glioblastoma multiforme (Grade IV), 4 anaplastic oligodendroglioma (Grade III), 2 anaplastic ependymoma (Grade III). Most of these specimens are currently available in our brain tumours tissue bank and stored in a -70°C freezer.

DNA was extracted from fresh tumour tissue using commercial extraction kits, according to standard protocol as suggested by the manufacturer's (QIAGEN Inc.) with minor modifications. Leukocytes from peripheral blood of normal patients were used to prepare normal DNA control.

Polymerase chain reaction (PCR) amplification

The oligonucleotide primers used for PCR amplification were purchased from Biobasic Inc. (Canada). PCR was performed using the oligonucleotide primers and amplification condition for exon 5-8 of *p53* gene as previously reported by Sarkar et al[8]. The PCR reaction mixture consisted of 1 X PCR buffer, 200μM of dNTP, 1.5 or 2mM $MgCl_2$, 1.0μM (50 pmol) of each primer and 2.5 units of Taq DNA polymerase and 50 ng of genomic DNA in a total 100μl reaction volume. Amplification was carried out in Thermal Cycler (Biometra, Germany) for 35 cycles. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. After the last cycle of amplification, the extension continued for an additional 7 min at 72°C. The PCR products were resolved on 2% agarose gels and stained with ethidium bromide.

Single-strand conformation polymorphism (SSCP)

SSCP analysis was performed according to a slight modification of the method of Sarker *et al*[8]. 5μl of each PCR products was added to 5μl of sequencing stop solution (95% formamide, 0.5 M EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and heated at 94°C for 3 minutes to denature the double-stranded DNA into a single-stranded fragment. The samples were chilled on ice before loading onto the gel. The denatured samples were loaded in a 16 x 20 cm, 0.75mm, 0.5X MDE gel (FMC BioProducts, U.S.A) which contained 5% glycerol and 1X TBE buffer (90mM Tris, 90mM boric acid, 2mM EDTA). The fragments were electrophoresed at 10 watts constant for 4.5 hours using Dcode Universal mutation Detection System (Bio-rad Laboratories U.S.A). The Dcode system was placed in a 4°C cold room. Seven liter of 1X TBE buffer was added and it was allowed to cool to 10°C. The Dcode temperature controller was set at 10°C to maintain the desired buffer temperature. After electrophoresis process was completed, the gels were stained with silver-staining solution.

Direct Sequencing Analysis

The samples that showed abnormal mobility in the SSCP analysis were isolated and run on 2% agarose gels and purified using a Geneclean II kit (Bio 101 Corp., La Jolla, CA). The sequencing was performed by the dideoxy chain-termination method using the same primers as in the PCR. Sequencing reactions was also carried out using the Ampli Cycle Sequencing kit (P.E. Applied Biosystem, U.S.A) and the ABI Prism Dye Terminator Cycle Sequencing (P.E. Applied Biosystem, U.S.A) on an ABI 3100 automatic DNA sequencer (P.E. Applied Biosystem, U.S.A).

Results

A total of 33 gliomas were examined for mutation in exons 5 to 8 of *p53* gene by PCR-cold SSCP. Specimens that showed electrophoretic mobility shift compared with the wild type DNA were considered to contain a mutant *p53* gene. The overall frequency of *p53* gene mutation was identified in 33% (11 of 33 gliomas) by PCR-cold SSCP (Figure 1 and 2) and subsequently confirmed by DNA sequencing (Figure 3). The results of this study are summarized in Table I. Significant electrophoresis mobility shift was detected in 2 of the 8 juvenile pilocytic astrocytoma grade I (25%), in 1 of the 3 astrocytoma grade II (33%), in 3 of the 9 anaplastic astrocytoma grade III (33%), in 3 of the 6 glioblastoma multiforme grade IV (50%), in 1 of the 4 anaplastic oligodendroglioma (25%) and the only pleomorphic xantoastrocytoma. The majority of mutations that we identified were localized in exon 7 (45.5%, 5 of 11) and exon 8 (36.4%, 4 of 11). There were 2 (18.1%) mutations in exon 6. No mobility shift or mutation was detected in exon 5. The spectrum of the eleven p53 mutations in these series of tumours included 7 mutations (63.6%) were single base pair substitution (point mutation) resulting in 5 missense mutations, 1 nonsense mutation and 1 silent mutation (Figure 4). Three mutations (27.3%) were insertions and 1 (9.1%) was deletion. In our investigation, transitions accounted for 57.1% (G:C --> A:T, 4 cases) and tranversions were 42.9% (G:C --> T:A, 1 Case; A:T --> C:G, 1 case; A:T --> T:A, 1 case).

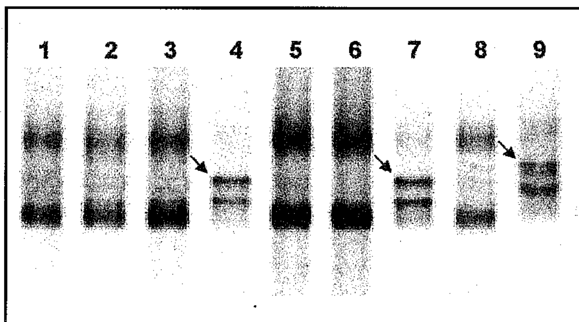

Fig. 1: PCR-SSCP samples run at 10°C on the Dcode system shows the pattern of exon 7 of *p53* gene. Normal specimen (wild type) shows in lane 1 and 8. Lane 2, 3, 4, 5, 6, 7 and 9 represent corresponding tumor specimen. The abnormal SSCP pattern shown in the tumor samples in lane 4, 7 and 9 are indicated by arrows.

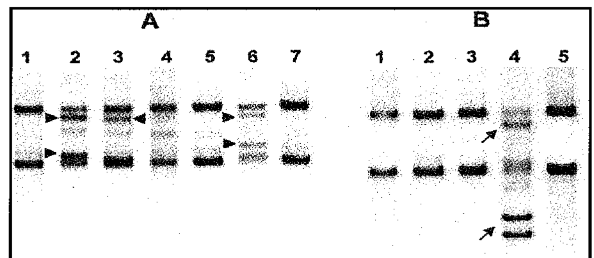

Fig. 2: Typical PCR-SSCP gel shows the pattern of exon 8 of *p53* gene.
A : Lane 1 and 7 represent normal samples (wild type) whereas lane 2, 3, 4, 5 and 6 represent tumor specimens. The electrophoretic mobility shifts of SSCP band is indicated by arrows in lane 2, 3 and 6.
B : Lane 1 and 5 represent normal samples (wild type) whereas lane 2, 3 and 4 represent tumor specimens. Arrows indicate aberrant bands in lane 4.

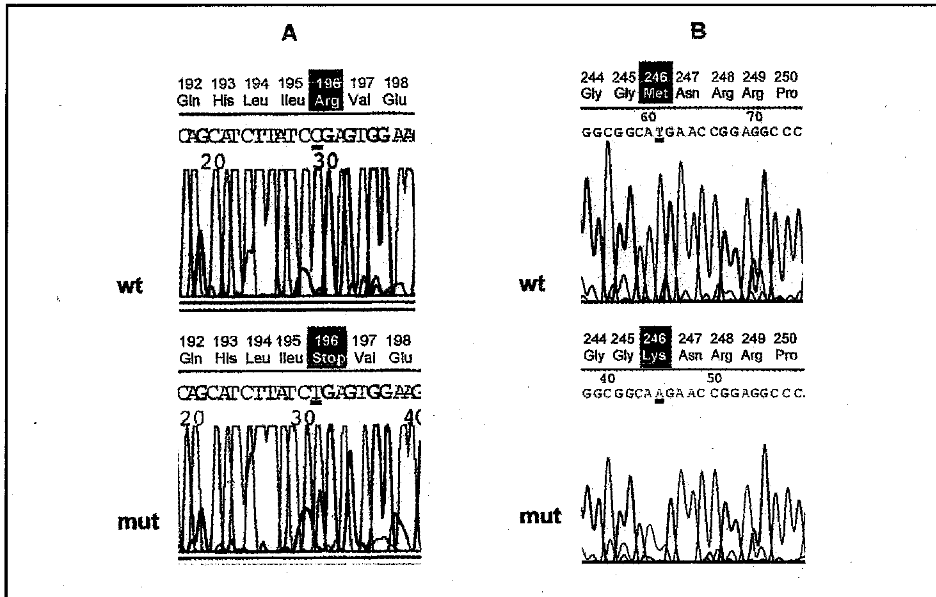

Fig. 3: Examples of electropherograms of DNA sequencing analysis of *p53* gene mutation. Mutant cases (mut) show substituted bases compared with wild-type cases (wt). A : C→T transition at codon 196 (exon 6) in case 27 and B : T→A transversion at codon 246 (exon 7) in case 10.

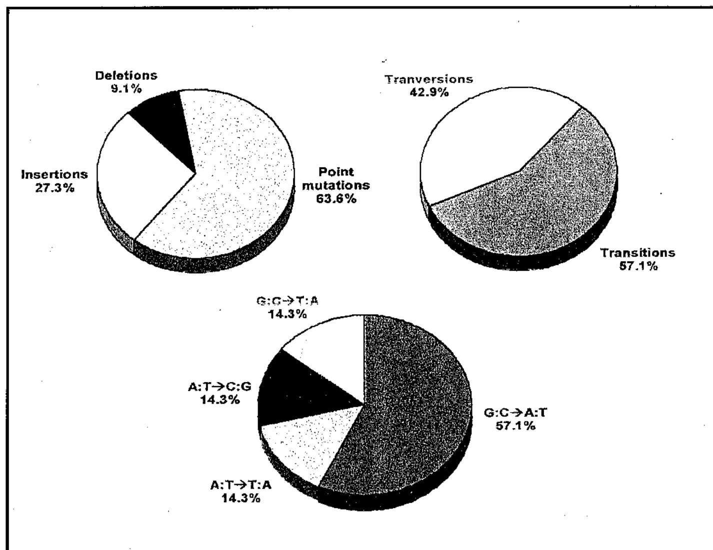

Fig 4: *p53* gene mutational spectrum in gliomas seen in Malay patients

Table I
Pattern of *p53* gene mutations in Gliomas from Malay patients

Case	WHO* grade	*p53* mutation				
		SSCP shift #	Codon	Nucleotide change	Amino acid Change	Type of mutation
1	JPA (I)	exon 7	249	AGG→ AAG	Arg→Lys	Transition (G:C to A:T)
2	JPA (I)	exon 8	265	CTG→ TTG, silent	Leu→Leu	Transition (G:C to A:T)
3	JPA (I)	–	–	–	–	–
4	JPA (I)	–	–	–	–	–
5	JPA (I)	–	–	–	–	–
6	JPA (I)	–	–	–	–	–
7	JPA (I)	–	–	–	–	–
8	JPA (I)	–	–	–	–	–
9	A II	–	–	–	–	–
10	A II	exon 7	246	ATG→AAG	Met→Lys	Transversion (A:T to T:A)
11	A II	–	–	–	–	–
12	PXA II	exon 8	273-274	CTGG, 4 bp-deletion	Frameshift/Deletion	
13	AA III	exon 8	266	GGA→ GGCA, 1 bp-insertion	Frameshift/ Insertion	
14	AA III	–	–	–	–	–
15	AA III	exon 8	265	CTG→CGG	Leu→Arg	Transversion (A:T to
16	AA III	–	–	–	–	–
17	AA III	–	–	–	–	–
18	AA III	–	–	–	–	–
19	AA III	–	–	–	–	–
20	AA III	–	–	–	–	–
21	AA III	exon 6	197	GTG→TTG	Val→Leu	Transversion (G:C to
22	GBM IV	–	–	–	–	–
23	GBM IV	exon 7	228	GAC→ GCAC, 1 bp-insertion	Frameshift/ Insertion	
24	GBM IV	–	–	–	–	–
25	GBM IV	–	–	–	–	–
26	GBM IV	exon 7	249	AGG→AAG	Arg→Lys	Transition (G:C to A:T)
27	GBM IV	exon 6	196	CGA→TGA	Arg→stop	Transition (G:C to A:T)
28	AO III	–	–	–	–	–
29	AO III	exon 7	231	ACC→ ACGC, 1 bp-insertion	Frameshift/ Insertion	
30	AO III	–	–	–	–	–
31	AO III	–	–	–	–	–
32	AE III	–	–	–	–	–
33	AE III	–	–	–	–	–

* WHO grade : JPA I = Juvenile pilocytic astrocytoma grade I; A II = astrocytoma grade II; PXA II = pleomorphic xantoastrcytoma II; AA III = anaplastic astrocytoma grade III; GBM IV = glioblastoma multiforme grade IV; AO III = anaplastic oligodendroglioma grade III; AE III = anaplastic ependymoma grade III. # SSCP shift = mobility shift on single strand conformation polymorphism (SSCP) analysis

Discussion

Our study is the first report of a mutational analysis of the *p53* tumour suppressor gene in Malay patients with human gliomas. We examined the *p53* gene mutation in a total of 33 gliomas by non-isotopic PCR-cold SSCP and sequencing analysis.

Mutations of the *p53* gene were detected in 2 of 8 (25%) juvenile pilocytic astrocytoma, 1 of 3 (33%), astrocytoma grade II, 3 of 9 (33%) anaplastic astrocytoma grade III, 3 of 6 (50%) glioblastoma multiforme grade IV, 1 of 4 (25%) anaplastic oligodendroglioma and the only pleomorphic xantoastrocytoma. These mutations occurred in codon 196, 198, 228, 231, 246, 249, 265, 266 and 273-274. When these mutations were compared with previously proposed hot spot (in codon 175, 196, 213, 248, 249, 273, 282) drawn from analyses of various neoplasms, four of the mutations were observed in the hot spot sites in codon 196, 249 and 273-274[16]. Similar results have been reported in other studies[17,18,19]. Among the four hot spot codons, two were identified at a CpG sites in codon 196 and 273 but not on codon 249[5,6,20]. The other seven mutations were located outside the hot spots, and mutations of codons 197, 228 and 231 have not previously been reported in gliomas so far. In the *p53* gene mutations database, which covers > 7500 mutations, missense mutation at codon 197 have been described in one case of breast cancer, with the GTG to TTG change, also found in our case[20]. The majority of mutations identified in our study were G:C to A:T transition (36.4%, 4 of 11). Previous studies of gliomas reported a higher prevalence of transition then transversion mutation in *p53*[5].

Mutations in codon 249 of *p53* gene are very rare in non-hepatic tumour. In our patients, we found mutations in codon 249 where the nucleotide change from AGG to AAG transitions (Arg --> Lys) while Wu *et al.*[17] reported the nucleotide change from AGG to AGT transversions (Arg --> ser) in glioblastoma multiforme. The G to T transversions at codon 249 are very common in hepatocellular carcinomas from South Africa, possibly related to aflatoxin B1 exposure, and are also the most common mutation in lung cancer. We also identified one case of pleomorphic xantoastrocytoma that had a deletion of 4 bp nucleotide (CTGG) occurred at known 'hot spot' at codon 273-274 in exon 7. The mutation at codon 273 has been reported previously as a hotspot for somatic alteration in gliomas[6,8,11,21]. In addition to codon 273, a high frequency of somatic mutation has been described in codon 175 and 248 of p53 in gliomas[6,8,11]. However no such mutations at codon 175 or 248 in our population probably due to our small series. The ATG-->AAG alteration at codon 246 resulting in methionine to lysine amino acid substitution, has been shown as a somatic genetic alteration in anaplastic astrocytoma grade III.22 However, in this study mutation at this codon observed in astrocytoma grade II. C to T transition was identified in codon 196 resulting in a change from arginine to a stop codon (CGA-->TGA) as similarly found by the Taiwanese groups[23].

Specific mutations of the *p53* tumor suppressor gene in astrocytic gliomas have been reported from several parts of the world, but to the authors' knowledge to date the status of this gene has not been studied in these tumours in Malay patients. The frequency of *p53* gene mutations observed in this study suggested that it could be one of the genes involved in the genesis of gliomas in Malay patients. Our findings showed the major hot spots at codon 249 and 273 of *p53* gene were also identified in the Malay patients with gliomas. The recent observations in molecular mechanisms of gliomas tumourigenesis may be useful molecular markers for diagnostic, prognostic, and therapeutic implication of Malaysian brain cancers.

Acknowledgements

This research was supported by grant from The Malaysian Toray Research Foundation Grant (No: 380/0500/5051). This research was done as a fulfillment for the Master of Science (Human Genetic), Universiti Sains Malaysia, for Abdul Aziz Mohamed Yusoff.

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