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IDENTIFICATION OF P53 TUMOR SUPPRESSOR GENE ALTERATIONS IN HUMAN GLIOMA USING SSCP-PCR TECHNIQUE

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Abstract

The P53 is the most commonly altered gene in cancers, which is located on chromosome 17p. Because of it is important role in the cell cycle it considered as the guardian of the genome that maintains genome integrity. In this study we analyzed DNA from 15 surgical specimens of human glioma for mutations in the p53 gene along exons 6-9 using single-strand conformation polymorphism analysis of polymerase chain reaction product (PCR-SSCP). The frequency of P53 gene mutation in gliomas examined was 26.6% (4 of 15). All 4 mutated cases were in exon 6 and no mutation was observed in exon 7,8 and 9. The results suggest the involvement of P53 gene mutation in Glioma and it might play an important role in the tumorigenesis process of patients with brain tumor in preliminary study in Duhok province.

KEY WORDS: *Brain Tumors, Glioma, SSCP-PCR, P53*

Introduction

Cancer is an abnormal growth of cells caused by multiple changes in gene expression leading to dysregulated balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity and, if untreated, death of the host (Ruddon, 2007). One of the most devastating central nervous system (CNS) pathologies is brain cancer , in children they are considered the second commonest form of cancer and the sixth commonest form in adults (Fomchenko and Holland, 2005 ; Smith and Ironside, 2007). With respect of their origin there are two groups of brain tumors, primary brain tumors originate in the brain and metastatic brain tumors, which develop elsewhere in the body and spread to the brain (Zupanska *et al.*, 2002 and Barlow and Ironside, 2009). Glial tumors/ glioma refer to neoplasms of the neuroglial cells, which are the most frequent primary brain tumors accounting for approximately 70% of all brain neoplasms (Chosdol *et al.*, 2002 and Gomes *et al.*, 2007). Gliomas account for about 2% of the malignant tumors in adults and malignant gliomas are the leading cause of CNS tumor related death (Phatak *et al.*, 2002). Histologically, gliomas are divided into several entities, with astrocytic tumors being the most prevalent type, followed by oligodendroglial and mixed oligoastrocytic tumors (Gomes *et al.*, 2007). Glial tumors, such as astrocytoma, glioblastoma multiforme (GBM) and oligodendroglioma, are the most common primary tumors in the CNS (Isa *et al*., 2003). As is the case for all human cancers, the genes that are altered in CNS tumors can be grouped into two general categories: oncogenes and tumor suppressor genes (TSGs) (Osman, 2005). Primary human tumors are frequently associated with genetic abnormalities, including the deletion of TSGs and the activation of oncogenes (Quan *et al.*, 2005). The activation of oncogenes by duplication or amplification, point mutation, and rearrangement, and the inactivation of TSGs by mutations, physical loss, or deletion are common genetic alterations in nervous-system tumorigenesis (Haberland, 2007). An important TSG is the p53, which is located on chromosome 17p and consists of 11 exons, it is considered as the guardian of the genome that maintains genome integrity (Hupp *et al.*, 2000; Preusser *et al.*, 2006). The p53 dysfunction is the frequent event occurring in gliomas (Zupanska *et al.*, 2002).

The theory of SSCP-PCR is that the primary sequence and the length of a single strand DNA fragment determine its conformation when it is resolved in polyacrylamide gel. Even single base difference can cause different secondary conformations and thus result in different migration rates of the DNA strands (Bartlett and Stirling 2003).

In this study we aim to determine p53 mutation in human giloma using Single strand

conformation polymorphism (SSCP-PCR technique.

Materials and Methods

Sample collection

Fiftteen samples of fresh brain tumor tissues were obtained by the Staff Neurosurgeons at Duhok Accident and Emergency Hospital (Teaching) patients harboring brain tumors; the samples were placed immediately in sterilized cup filled with absolute Ethanol. Samples of tumor tissues were subjected for histopathological diagnosis and were all diagnosed as glioma. In all cases, part of the affected tissues were taken and considered as a source of mutant DNA.

Venous blood samples, 5ml amount, were drawn from the same patients and placed in sterilized tube containing EDTA. The leukocytes of the collected blood were considered as a source of normal DNA.

The study was conducted in Scientific Research Center, Faculty of Science, University of Duhok. Both tumor tissues and normal blood samples were transferred to laboratory Scientific Research Center and stored at -20°C until analysis.

Genomic DNA were extracted (from both tumor and blood) by Proteinase K digestion and Phenol: Chloroform extraction method that Modified by Bass *et al.,* (1984) was used for extracting DNA from the patients' blood and the method described by Maniatis *et al.,* (1982) was used for extracting DNA from brain tumor samples. NanoDrop 1000 Spectrophotometer was used for the determination of the DNA concentration and purity.

Primers for PCR

The primers used were oligonucleotides complementary to the sequence flanking the exon / intron junctions of exons 6-9 for P53 gene (Kovach *et al* 1991). The sequence of each primer is shown in Table 1:

Table (1): Sequences of primers exon 6-9 for P53 gene

Exon6 forward **reverse**	5′- CTCTGATTCCTCACTG-3′ 5′-ACCCCAGTTGCAAACC-3′	160bp	57°C
Exon7 forward **reverse**	,5′-TGCTTGCCACAGGTCT-3′ 5′-ACAGCAGGCCAGTGT-3′	210bp	65°C
Exon8 forward **reverse**	5′-AGGACCTGATTTCCTTAC-3′ 5′-TCTGAGGCATAACTGC-3′	245bp	57°C
Exon9 forward **reverse**	5′-TATGCCTCAGATTCACT-3′ 5′-ACTTGATAAGAGGTCC-3′	149bp	56°C

Polymerase chain reaction

Genomic DNA (100 ng) was used for amplification in 25 µl of reaction mixture. A Cinagen PCR ready master mix kit was used for the amplification as follow:

The composition of the reaction mixture used for all exons was: 12.5 uL Master mixture, 8.5 uL D.D.W, 1uL Forward primer, 1uL Reverse primer 1uL, and 2 uL DNA sample. PCR conditions for exon 6 and 8 were 94°C for 5 min, 30 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 30 s and a final cycle of 72°C for 10 min. The condition for exon 7 was 94°C for 5 min, 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 10s, and a final cycle of 72°C for 10 min. The condition for exon 9 was 94°C for 5 min, 30 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 15 s, and a final cycle of 72°C for 10 min.

Single strand conformation polymorphism analysis

Amplification of PCR products were checked by 2% agarose gel electrophoresis. 4uL PCR product diluted in 10uL formamide and 3uL loading dye. The mixture denatured at 90 C^0 for 5 minutes, then put the samples immediately on the ice, and finally products were loaded onto 6% denaturating polyacrylamid gels, and DNA fragments were visualized by silver staining kit (Promega, Madison, Wis) as described by the supplier. Silver – stained gels were scanned to

capture digital images of the gels after air drying.

Results and discussion

In this study 15 samples of gliomas were screened for the presence of mutations in P53 gene along exon 6-9 using PCR-SSCP technique. Amplicons obtained by exon 6, 7, 8 and 9 of the P 53 gene were subjected to PCR-SSCP analysis. A shift in band mobility or abnormal bands considered as positive for the mutation (Phatak *et al.*, 2002). Among 15 samples of gliomas were checked for mutations only four tumor DNAs showed an altered pattern in PCR-SSCP analysis, which is in additional band in exon 6 moving differently from normal bands (figure 1; table 2). None of the other exons showed any shifted band or additional one.

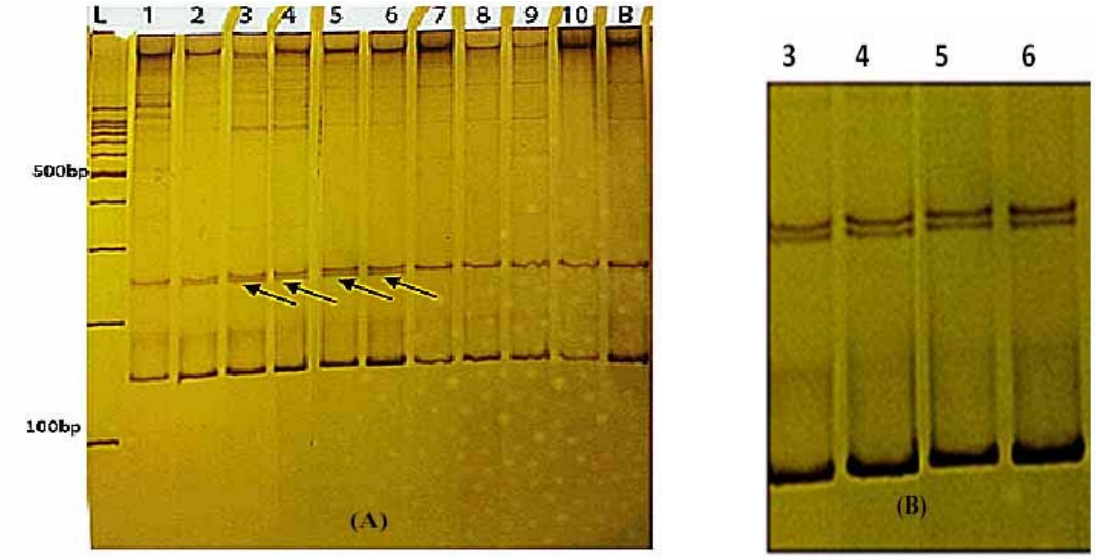

Figure (1): Typical PCR·SSCP gel shows the pattern of exon 6 of *p53* gene. (A) Lane L represents 100bp DNA ladder, lane 1-10 represent Tumor DNA, lane B represent control, black arrows represent the electrophoretic mobility shifts of SSCP band in lane 3,4,5 and 6.(B) Represent shifted SSCP bands.

Table (2): The results obtained from SSCP- PCR of *p53* gene

Glioma sample number	SSCP Shift/abnormal band			
	Exon6	Exon7	Exon8	Exon9
1	-	-	-	-
2	-	-	-	-
3	+	-	-	-
4	+	-	-	-
5	+	-	-	-
6	+	-	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	-	-	-	-
14	-	-	-	-
15	-	-	-	-
Total mutated samples	4	0	0	0

(+)= positive for the mutation, (-) negative for the mutation

The p53 is mutated in a wide variety of human neoplasms including Glioma. (Patt et al., 1996; Jafri *et al.,* 2003 and Cui *et al.,* 2008). Allelic loss of chromosome 17p and mutation of p53 have been observed in approximately one-third of adult astrocytic tumors of WHO grades II, III, and IV, suggesting that inactivation of p53 is an early event in the formation of grade II tumors (Sarkar et al., 2002 and Ohgaki et al., 1993).

According to a study conducted by Phatak et al., (2002), which they have applied PCR-SSCP analysis on 44 Indian patients with glioma they found 13.6% of them carry the mutation in the P53 gene. However In the present study the frequency was higher 26.6% (4 of 15 samples) using the same method. Phatak et al., (2002) found that the p53 mutation occurred in exon 5, 7, and 8. While in our study the mutation is restricted to exon 6, this suggest that the studied samples in Kurdistan population carry different type of p53 mutation from other population that needs further investigation such as sequence analysis to identify which codon is affected by these mutations. Additionally Cui *et al*., (2008) found p53 gene mutations were 41.5% (17 of the 41 specimens) of gliomas. More over Jafri *et al.* (2003) identified that 18.1% of glioma samples that they investigated were carry the mutation of exon 6, this support our results that there is mutation occurred in exon 6 as well rather than the rest exons.

The deference of p53 mutations occurrence in different populations might be due to the difference in the exposure to the amount of causative agents of the mutation in different geographical area.

The p53 gene is one of the most important human tumor suppressor gene which affects both cell growth and cell death. Experimental evidence suggests that the function of p53 could be modulated at various levels – gene structure, expression, level of mRNA and protein, protein conformation and post-translational modification. Apart from point mutations, alteration at any of these levels could also affect the function of the protein and hence could lead to tumor development (Keshava *et al.,* 2010). In our previous study using random amplified polymorphic DNA (RAPD –PCR) we identified the presence of single and multiple genetic alterations in brain tumor cells (Ameen *et al.,* 2012). The results obtained in the present study suggest that the P53 mutation might be one of the factors that led to occurrence of this genomic instability in analysed brain tumor samples in particular glioma in Duhok \ Kurdistan population. More over the results suggest an involvement of p53 mutations in the gliomas and may be a reason for its tumorigenesis and malignancy in Duhok \ Kurdistan patients with brain tumor.

Human brain tumors are one of the most difficult to manage and treat. Therefore, development of novel approaches is essential to manage and treat these tumors. Therefore we recommend further investigation by immunohistochemistry and molecular methods as well to survey the occurrence of p53 gene mutation more accurately.

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