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RESEARCH ARTICLE

P53 GENE CODON 72 POLYMORPHISM GENOTYPING IN MENINGIOMA AMONG SUDANESE PATIENTS

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ABSTRACT

Background: Meningiomas predominantly benign tumors that may result from an adverse effect of cranial irradiation and trauma. They account for 20% of all primary intracranial neoplasm. The incidence is likely to be much higher, since many benign meningiomas do not produce symptoms. The p53 tumor suppressor is a key regulator of cell cycle progression, such that its inactivation promotes increased cell growth and tumor genesis.
Objectives: The study design to characterize the p53 codon polymorphism in Sudanese memimgioma patients & to correlate the p53 genotype with biological characteristic of the tumor.
Material and methods: The study was conducted at Al-Shaab Teaching Hospital at The National Center for Neurologic Sciences (NCNS) during the period September 2009 and March 2011. It includes cranial meningioma Sudanese patients that were diagnosed based on radiology and histology. 70 meningiomas' tissue samples were taken for DNA extraction. The amplification was carried out using PCR.
Results: The genotyping analysis of 70 cases for p53 alleles revealed Arg/Arg was reported in 70% of the cases (30) whereas Pro/Pro seen in 17 (24.3%) and Arg/Pro was reported in only 4 (5.7%)

INTRODUCTION

The Meningiomas are one of the commonest intracranial tumors and account for 20% of all primary intracranial neoplasms. However, the true incidence is likely to be much higher, since many benign meningiomas do not produce symptoms. The frequency of meningiomas has been the topic of relatively few reports, and those provide information from either hospital- or population-based studies. Hospital-based brain tumor series indicate that the incidence is approximately 20% of all intracranial tumors, a figure derived from the combined results of several large series (Nayar *et al*., 2010; McGovern *et al*., 2009). Genetic analysis of meningiomas has identified several important candidates. Of those Neurofibromatosis 2 (*NF2*), Tumor Suppressor in Lung Cancer-1 (TSLC1) and P53 are the most important. Loss of the neurofibromatosis 2 gene (*NF2*) on chromosome 22q is the best characterized alteration in meningioma. The importance of *NF2* loss to the molecular pathogenesis of meningioma is underscored by the seminal observation by Giovannini and associates, in which mice with leptomeningeal inactivation of *NF2* develop meningioma (Kalamarides *et al*., 2002; Kalamarides *et al*., 2010; Goutagny *et al*., 2010). Human p53 is a nuclear phosphoprotein of MW 53 kDa, encoded by a 20 –kb gene containing 11 exons and 10 introns, which is located on the small arm of chromosome 17. It belongs to a highly conserved gene family containing two other members, p63 and p73.Wild – type p53 protein contains 393 amino acids and is composed of several structural and functional domains. N terminus containing an amino – terminal domain (residues 1-42) and proline- rich region with multiple copies of the pxxp sequences (residues 61-94 where x is any amino acid), a central core domain (residues 102-292) and a C- terminal region (residues 301- 393) containing an oligmerization domain (residues 324- 355), strongly basic carboxyl- terminal regulatory domain (residues 363- 393), a nuclear localization signal sequence and 3 nuclear export signal sequence Recent studies have highlighted that the p53 codon 72 polymorphism plays a crucial role in modulating wild-type p53 apoptotic capacity, and thus may influence the biological characteristics of the tumors and hence their natural history in a sense of the recurrences and the response to chemotherapy. Previous

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studies indicate that breast cancer patients with the Pro/Pro variant may be less sensitive to anthracycline-based treatment than those with the Pro/Arg or Arg/Arg variant and suggests that analysis of P53 codon 72 polymorphism may provide a simple predictive marker for selecting the right breast cancer patients to anthracycline-based neoadjuvant chemotherapy in clinical setting. As a tumor suppressor, p53 is essential for preventing inappropriate cell proliferation and maintaining genome integrity following genotoxic stress. Following various intracellular and extracellular stimuli, such as DNA damage (by means including ionizing radiation, UV radiation, application of cytotoxic drugs or chemotherapeutic agents, and infectious virus), heat shock, hypoxia, and oncogene over expression, wt p53 is activated and emerges as a pivotal regulatory protein which triggers diverse biological responses, both at the level of a single cell as well as in the whole organism (Jallepalli *et al*., 2001).

MATERIAL AND METHODS

Study setting and patients

This is a descriptive cross-sectional study conducted at Alshaab Teaching Hospital during the period September 2010 and March 2011. All Sudanese patients attending Al-Shaab hospital during the period of the study and diagnosed clinically and/or histologically as intracranial meningioma were included in the present study. Approval was taken from the Ethical Review Board at the national center for neurological sciences (NCNS) and verbal consents from all patients were obtained.
Samples collection & processing:

Fifty-nine meningiomas' tissue samples were taken in sterile containers, that contain RBMI 1640 media and processed for DNA extraction.

Sample preparation

Around 200 mg from each tumor was taken in Petri dishes and minced using surgical blade into small pieces. The DNA extraction was done, using guanidine chloride method. Fifty µl of previously extracted DNA were taken in a clean eppendorf tube, then 30 µl of proteinase K were added, the sample was then incubated at 65 c° for overnight, vortexed and 200µl of pre chilled chloroform were added, the mixture was then vortexed and centrifuged at 12000 RPM for 10 minutes, the upper layer was collected in a clean eppendorf tube, then 400 µl pre chilled ethanol was added, and incubated at -20C over night. Next day the tube was centrifuged at 12000 RPM for 10 minutes, after that supernatant was discarded, the collected DNA was washed with 300 µl 70% ethanol, then the tube was centrifuged at 8000 RPM for 5 minutes, then discarded the supernatant. The deposited DNA was air dried and 50 µl deionized water was added to purify DNA. The purified DNA was then incubated at 4 ^{O}C overnight, gel electrophoresis was done for extracted DNA to assess the quality of the extracted DNA processing Extracted meningioma DNA was brought form -20ºC, and kept on ice rack for processing, at the same time stock primers, dNTPs, and buffer were brought at room temperature (RT) and kept on ice rack for thawing and sterile deionized water was brought out from refrigerator and aliquoted on 1.5 ml tube and kept as above.

Primer preparation

Each of the forward and reverse primers were prepared by adding10µl of each stock primer (100 µM) to 90µl deionized water, the solution was mixed carefully using sterile tips to ensure its homogeneity.

P53 sequence

Arg F 5 tcc ccc ttg ccg tcc caa 3
Arg R 5 ctg gtg cag ggg cca cgc 3
Pro F 5 gcc aga ggc tgc tcc ccc 3
Pro R 5 cgt gca agt cac aga ctt 3
preparation of deoxynucleotides (dNTPs)

Deoxynucleotides were prepared by adding 10µl of each nucleotides (total volume 40), in 60 µl of sterile deionized water to a final concentration of 10 mM. The mixture was vortexed to collect any dNTPs from the tube surface in the button of the tube.

Master Mix preparation

Master mix (MM) was prepared using P53- F and P53- R, according to the number of samples to be processed with an extra one more sample to compensate pipetting errors. PCR reagents, were added in the order listed on the worksheet, adding water first and Taq polymerase last. 23µl of MM was added into each tube, all reagents were kept in a frozen-cryo-rack. Caps were closed tightly and the PCR tubes were placed in the loading area. To avoid contamination, the tips were always changed and the avoidance of touching the side tube and capped was recommended.

PCR optimization

Sample with known positive to p53 gene alleles was first amplified by PCR. After successful amplification the rest of the samples were then analyzed.

Running the PCR

All general PCR considerations were applied including the following:-

Samples tubes were placed into the wells of thermal cycler according to the manufacture's recommendation. The thermal cycler was programmed for the appropriate cycle condition for the P53 gene.

Before placing the PCR tubes into thermal cycler, the PCR tubes were given "Quick–spin" to ensure that all reagents and samples were drawn from side of tubes. After cycling, before opening the PCR tubes, the PCR tubes were given "Quick-spin".

PCR amplification of P53

In a 0.5 ml PCR tube the following solutions were placed in a total volume of 25 µl, for 1X reaction, 2.5µl PCR buffer with concentration of 200mM, Magnesium chloride 1.5 µl with final

concentration of 50mM, dNTPs 1µl final concentration of 10mM, Taq polymerase 0.3µl with final concentration of 500 unite, extracted meningioma DNA 2µl (Approximately 200 ng) repeated cycles 35 times using a PCR thermal cycler (TC-3000) at 95 C◦ for 3minutes, 94 for 20-20 seconds, 65 for 45 seconds, 72 for one minute, 57 for 45 seconds, and at 72 for 10 minutes. Using 2% Agarose gel electrophoresis amplified PCR product were visualized

Agarose gel electrophoresis

Agarose gel electrophoresis is a rapid technique used to identify, quantify, and purify nucleic acids.. Using sensitive balance 0.7 gm from Agarose powdered were prepared, then 35 ml of working buffer was added (28 ml ddwater +7ml from 5X TBE buffer), the mixture were placed in microwave (NIKAI-Japan) for 2 minutes, then the bottle which contain the mixture was placed at room temperature just to be warm, 0.9 µl of Ethedium bromide was added to the mixture with gentle mixing, then the mixture of Agarose, TBE buffer, and Ethedium bromide were poured into gel electrophoresis tray which contains two sides for combs, each comb with eight tips. After the gel is formed the combs were removed leaving sixteen wells at each side of the gel. The running buffer was prepared using 4ml of TBE buffer, 400 ml of distilled water. The gel electrophoresis tray which contains the gel was placed in the gel electrophoresis tank, then the tank was filled with the running buffer to the mark maximum. A suitable piece of parafilm was added near the tank, then 2µl from the loading dye were added for each 5µl PCR product, then the loading dye and PCR product were mixed and placed in the gel wells, DNA ladder was placed at the left well, then the tank was connected to separating machine. The gel was placed into gel documentation box, and was firstly adjusted using white light followed by UV light to visualized the DNA bands.

Preparation of tris-borate EDTA solution

The working solution of 1X TBE was prepared from the stock solution (1/Liter) which contains 27.5 gram Tris base, 14 gram boric acid, and 0.93 gram EDTA. It is used for agarose gel preparation and as a running buffer for electrophoresis.

Preparation of DNA Loading Dye Solution

The dye was prepared from 10 ml of pure glycerol, 0.25 gm bromophenol blue and 0.4 ml EDTA. It was then mixed and stored at 4ºC.

Preparation of DNA ladder

DNA ladder was prepared for electrophoresis using 4 µl from DNA ladder and 20 µl loading dye. *PCR products was separated by agarose gel electrophesisis. Samples were loaded on 2% Agarose stained with Ethdeium bromide.*

Data processing and statistical analysis

Data were analyzed using SPSS 13 software with reference *P.value* of 0.05 was considered statistically significant.

RESULTS

A total number of 70 cases were enrolled into the study, 46 (65.7%) of them were women compared with 24 (34.3%) men. The histological grading done according to WHO graded (WHO, 2007) revealed that grade I constituted 82.9% that represented 58/70, most of them were reported between females 41/58 (70.7%), followed by grade II that constituted 14.3% (4 cases in women compared with 6 cases in men) where as grade III was reported in only 2.8%, one case in women and one case in men (table 1). The genotyping analysis of p53 alleles revealed alleles Arg/Arg was reported in 70% of the cases (30 cases among women compared with 19 cases among men) whereas Arg/Pro was reported in only 4 (5.7%) cases (2 cases among women & 2 cases among men), and Pro/Pro seen in 17 (24.3%) cases (14 case among women & 3 among men) (table 1).

Using histopathological examination, fibrous meningioma was found as the most common type that represent 45.7% (32/70) followed by meningiothelial, atypical & clear cell meningioma (table 2). The histology analysis of the tumor with the genotype Arg/Pro revealed that 2 psammomatous meningioma and one metablastic, and secretary type. The genotype Pro/Pro was seen in 6 fibrous, 4 meningiothelial, 2 psammomatous, and one in angiomatous, transitional and atypical types The correlation between histopathology grade and the p53 alleles showed that Arg/Arg type was present in 26 tumors of grade 1, 8 tumors in grade 11, and 2 tumors in grade 111. Arg/pro type was present in 4 tumors with grade 1, whereas Pro/pro type was present in 14 tumors with grade 1 and, 2 tumors with grade 11 (Fig.1).

Table 1. Distribution of the p53 codon 72 polymorphism among meningioma patients according to gender and WHO grade

P53 alle	Women				Men			
	WHO grade				WHO grade			
	G I	G II	G III	Total	G I	G II	G III	Total
Arg/Arg	26	3	1	30	13	5	1	19
Arg/Pro	2	0	0	2	2	0	0	2
Pro/Pro	13	1	0	14	2	1	0	3
Total	41	4	1	46	17	6	1	24

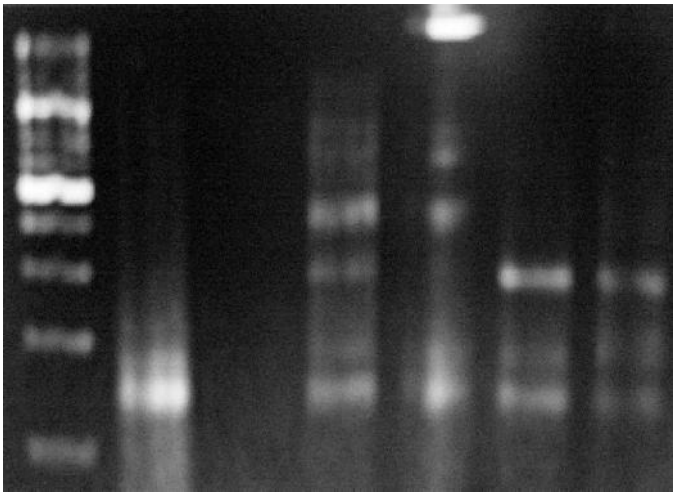

Fig. 1. gel electrophoresis for p53 alleles (Arg/Pro)

Table 2. Association with polymorphisms in p53 alle and histological type of meningioma

	P 53 alle				Gender		
	Arg/Arg	Arg/pro	pro/pro	Total	Women	Men	Total
Angiomatuos	1	0	1	2	0	2	2
Fibrous	26	0	6	32	24	8	32
Metablastic	0	1	0	1	0	1	1
Psmmomatous	1	2	2	5	4	1	5
Transional	7	0	2	9	8	1	9
Atypical	8	0	1	9	4	5	9
clear cell	0	0	1	1	0	1	1
meningiothelial	5	0	4	9	5	4	9
secretary	0	1	0	1	1	0	1
anaplastic	1	0	0	1	0	1	1
Total	49	4	17	70	46	24	70

DISCUSSION

Loss of heterozygosity in p53 tumor suppresser gene has been studied in different types of brain tumors, but a little is known about p53 gene in meningioma known mostly as benign tumor especially in Sudanese patient. In this study we aimed to study the correlation between the p53 gene codon 72 polymorphism genotypes with the natural history of intracranial meningioma with special emphasis to the recurrence and/or progression. In fact previous studies of breast cancer among Sudanese women have suggested strong correlation between the p53 gene codon 72 polymorphism genotypes, and the aggressive biology of the tumors. In this study we analyze p53 codon 72 with the histological sub types of meningioma and WHO grades. There is significant association and co relation with Arg/Arg alleles with the fibrous sub type which considered as grade 1. However, classifying meningioma into two groups, the low and the high grades one can see that among the low grade which considered benign, 26 (out of 44) showed Arg/Arg, whereas in the cases of the high grade (considered as a malignant) only 9 (out of 12) cases were Arg/Arg. Although, several studies have investigated p53 at the gene level and at the level of protein in benign and malignant meningioma, its role remain controversial, particularly with regard to discrepancy between p53 over expression and gene mutation (Martuza *et al*., 1985).

In this study we examine 59 meningioma tumors (45 of the cases were benign and 11 of the cases were malignant), all of the benign tumors showed loss of heterozygosity of P53 gene using polymerase chain reaction amplification. In this study Arg/Arg alleles has been detected in 32 of the benign tumors, and Pro/Pro alleles were seen in 14 of the benign tumors, on the other hand only Arg/Arg alleles found in 9 of the malignant tumors. Several clinical and biological markers were tested for the prediction of the natural history of the meningioma (recurrence) such as histological subtypes, the grade of the tumor, the size of the tumor, and the site of the tumors, all turn to be of little predictive value for clinical course of the tumor. However, some reports suggested high rate of meningioma recurrences in atypical and malignant ones, yet in the present study the striking finding of high recurrent rate in even benign tumors strongly point toward other factors that were responsible about aggressive meningioma tumors even in benign meningiomas such as the genetic profile of the tumor.

This has been investigated in numerous type of tumors such as breast, bladder, and also in hematological malignancy (Fadl-Elmula *et al*., 2001). In the present study strong correlation was observed between the recurrences of the tumors and Arg/Arg alleles of P53, moreover, the multivariant analysis showed no what so ever correlation between the others variants (Pro/Pro), and (Arg/Pro). Moreover, in taking the co relation between the p53 allele and the recurrence, Arg/Arg alleles p53 is the most common genotype seen, being associated with recurrence in 47.2% of the cases, mostly the fibrous one. Looking for the follow up of the patients one may argue that the mean recurrence duration (21.43 months) was short and perhaps we need to follow our patients further before reaching final conclusions.

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