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P53 Suppressor Gene Tissue Microarray-based Protein Expression Analysis in Meningiomas

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Abstract. *Background/Aim: Meningiomas represent the main intracranial primary central nervous system (CNS) tumour in adults worldwide. Oncogenes' over-activation combined with suppressor genes' silencing affect negatively the biological behavior of these neoplasms. This study aimed to explore the impact of p53 suppressor gene expression in meningiomas' clinic-pathological features based on a combination of sophisticated techniques. Materials and Methods: Fifty (n=50) meningiomas were included in the study, comprising a broad spectrum of histopathological sub-types. An immunohistochemistry assay was applied on tissue microarray cores followed by digital image analysis. Results: p53 protein over-expression (high staining intensity levels) was observed in 27/50 (54%) cases, whereas the rest (23/50-/46%) demonstrated moderate to low levels of the protein. p53 over-expression was statistically significantly correlated to the mitotic index of the examined cases (p-value=0.001). Interestingly, the atypical/anaplastic group of histotypes demonstrated the strongest p53 expression rates compared to the others (p-value=0.001). Conclusion: p53 over-expression is observed in a broad spectrum of meningiomas. High expression levels lead to an aggressive biological behavior of the malignancy (combined with increased mitotic rates), especially in atypical and anaplastic sub-types that also have a high recurrence rate.*

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Neoplastic formation and malignant transformation are based on a cataract of abnormal intra- and extra-cellular reactions inside the corresponding epithelia (1). Up-regulation of proliferation combined with apoptosis inhibition critically influences the carcinogenetic process (2). Among genes that significantly modify crucial cell functions, p53 is one of high importance (3). Concerning cell cycle, p53 acts as a key regulator, stabilizing the genome domain and regulating its function. The corresponding gene -located on chromosome's 17 (Chr 17) short (p) arm at position 13.1 (17p13.1) –encodes for a 53 kDa nuclear phosphoprotein. p53 protein acts as a strong transcription/suppressor factor controlling cell proliferation. It is also implicated in different cell-signaling pathways that regulate cell cycle phases, programmed cell death, and DNA replication/repair (4). *MDM2*, a proto-oncogene (12q14.3) that encodes for a nuclear-localized E3 ubiquitin ligase, controls p53 protein expression levels in a p53-MDM2 auto-regulatory pathway. In fact, MDM2 binds

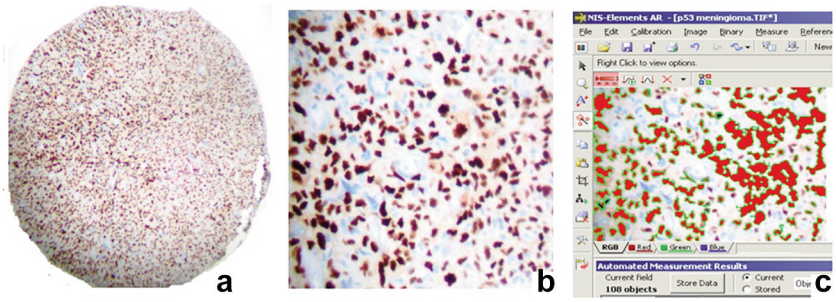

Figure 1. *p53 high-level expression in a meningioma tissue microarray spot (atypical histotype). A) A tissue core of 1.5 μm in diameter immunostained with the p53 antibody (original magnification 40×). B) p53 strong brown (light to dark) nuclear staining pattern. Note the high concentration of stained malignant nuclei (diaminobenzidine-tetrahydrochloride-DAB, original magnification 400×). C) Digital image analysis on the same atypical meningioma tissue core. Note the progressive, objective, and accurate measurement procedure. Reddish areas represent different expression levels on the p53 stained nuclei, whereas green lines encircle specific areas of interest (range=0-255 continuous grey scale RGB-based immunostaining intensity levels).*

directly to a p53 domain repressing its transcriptional activity and inducing p53 proteasomal degradation (5). The p53 protein is expressed at a low level in normal epithelia. p53 over expression -as a result of genetic events such as point mutations- is frequently identified in about 60% of malignancies of different histogenetic origin, including breast and colon adenocarcinoma, lung carcinomas, and also malignancies in the central nervous system (CNS) (6-9). A variety of immuno-histocytochemistry (IHC/ICC) assays are useful tools for detecting its aberrant expression.

Concerning CNS-derived neoplasias and malignancies, meningiomas represent the second most common brain tumour. In fact, they seem to be the most frequent intracranial primary CNS tumors in adults. Concerning their biological behavior, recurrence – especially in high grade cases - is associated with an aggressive phenotype affecting the response rates to surgery/radiation-applied therapeutic regimens (10). In the current research study, we detected and analyzed the different p53 protein expression patterns in a series of meningiomas and its potential impact on their corresponding pathological features using a combination of sophisticated techniques (tissue microarray platform and digital image analysis).

Materials and Methods

Study group. In the current research study, fifty (n=50) archival, formalin-fixed and paraffin-embedded meningioma tissue specimens were selected and used. The main purpose was a representation of a broad spectrum of histotypes. According to pathology classification histotypes, 12 meningotheliomatous, 12 psammomatus, 6 transitional, 5 fibrous, 2 angiomatous, 2 microcystic, 5 atypical, 5 anaplastic, and 1 papillary were recognized. Concerning the corresponding patients, 39 (78%) were female, and 11 (22%) male. The ethic committee consented to the use of these tissues in the 1st Dept of Pathology, Medical School University of Athens for research purposes, according to World Medical Association Declaration of Helsinki. The histochemical protocol was applied as described below: ''The selected tissue samples were initially fixed in 10% neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides of the corresponding samples were reviewed for confirmation of histopathological diagnoses. All neoplasias were classified according to the histological typing and grading criteria of World Health Organization (WHO) including also conventional mitotic indexes (mitoses per high power fields-HPF)'' (11, 12).

Tissue microarrays construction (TMA). Applying this method, we focused on areas of interest in the H&E-stained slides by a conventional microscope (Olympus BX- 50). The TMA protocol was implemented by using TMArrayer- 100 (Chemicon International, Temecula, CA, USA) device. All of the source blocks were cored, and 1.5 mm diameter tissue cylindrical cores were transferred from each conventional donor block to the recipient block. The final constructed TMA block contained 50 cylindrical tissue specimens. After 3 mm microtome sectioning and H&E staining, we observed microscopically that the final TMA density was 100% (full tissue microarray core adequacy) (Figure 1A).

Immunohistochemistry assay (IHC). Ready-to-use anti-p53 (clone DO7-DAKO, Glostrup, Hovedstaden, Denmark, dilution at 1:40) mouse monoclonal antibody was applied in the corresponding cases. The IHC protocol was applied in a similar way and modified as described in a previous analysis mediated by our study group (13). Nuclear predominantly and peri-nuclear staining pattern was considered acceptable for the marker regarding the TMA tissue spots, according to the manufacturers' data sheets (Figure 1B). Pre-analyzed breast cancer tissue sections expressing the protein were used as positive controls, according to manufacturer's guidelines.

Digital image analysis (DIA) assay. p53 protein expression levels were calculated in a quantitative way by measuring the corresponding staining intensity levels (densitometry evaluation) in the stained nuclei. The DIA protocol was applied by using a semi-automated analytical system (hardware: Microscope CX-31, Olympus, Melville, NY, USA, Digital camera, Sony, Tokyo, Japan; Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan). Areas of interest on every spot were detected (5 optical

Table I. *Clinicopathological parameters and total p53 expression results.*

Clinicopathological parameters		p53		*p*-Value
Meningiomas (n=50)		OE	MLE	
		27/50 (54%)	23/50 (46%)	
	n (%)	n (%)	n (%)	
Sex				0.981
Male	11 (22%)	7/50 (14%)	4/50 (8%)	
Female	39 (78%)	20/50 (40%)	19/50 (38%)	
Mitotic index (HPF)				
0-4	33/50 (66%)	14/50 (28%)	19/50 (38%)	**0.001**
>4, ≤19	10/50 (20%)	6/50 (12%)	4/50 (8%)	
≥20	7/50 (14%)	7/50 (14%)	0/50 (0%)	
Grade				0.091
I	36 (72%)	16/50 (32%)	20/50 (40%)	
II	8 (16%)	6/50 (12%)	2/50 (4%)	
III	6 (12%)	5/50 (10%)	1/50 (2%)	
Histotype				**0.001**
Atypical	5/50 (10%)	5/50 (10%)	0/50 (0%)	
Anaplastic	5/50 (10%)	5/50 (10%)	0/50 (0%)	
Papillary	1/50 (0.5%)	0/50 (0%)	1/50 (2%)	
Meningotheliomatous	2/50 (24%)	6/50 (12%)	6/50 (12%)	
Psammomatus	12/50 (24%)	5/50 (10%)	7/50 (14%)	
Transitional	6/50 (12%)	3/50 (6%)	3/50 (6%)	
Fibrous	5/50 (10%)	1/50 (2%)	4/50 (8%)	
Angiomatous	2/50(4%)	1/50 (2%)	1/50 (2%)	
Microcystic	2/50(4%)	1/50 (2%)	1/50 (2%)	

OE: Over-expression (high expression) staining intensity values ≤129; MLE: moderate-low expression staining intensity values >130 and ≤141. Statistically significant *p*-values are shown in bold.

fields at ×400 magnification) and filed in a digital database as snapshots. The corresponding measurements were extracted by constructing a specific macro for detecting and measuring p53 nuclear expression pattern in malignant cells, according to manufacturer's datasheet. Based on an algorithm, normal tissue sections (control) were evaluated independently and compared to the corresponding values in malignant tissue sections. A broad grey scale spectrum of continuous values (0-255) at the RedGreenBlue (RGB) analysis was observed for discriminating different protein expression levels (Figure 1C). According to DIA assay, immunostaining intensity levels decreasing to 0 represent a progressive over-expression of the marker, whereas values increasing to 255 show a progressive loss of its staining intensity. Total results and values are demonstrated in Table I.

Statistical analysis. For statistical analyses, descriptive and inferential techniques were applied. Statistics software package IBM SPSS v25 (SPSS Inc, Chicago, IL, USA) was applied. Quantitative variables were presented as mean±standard deviation, whereas the qualitative variables were presented in frequency tables. To evaluate the relationship between qualitative and quantitative variables, due to the small number of subjects in each group the nonparametric Mann-Whitney and Kruskall-Wallis tests were applied. To evaluate the relationship between independent qualitative variables, where appropriate, the control χ^2 for linear trend and the control of Fisher were applied. Statistical significance (p) was evaluated in pairs and differences <0.05 were considered statistically significant. Total IHC results and differences (p-values) are described in Table I.

Results

Based on the current applied digital expression analysis, the examined immunostained meningioma tissue microarray cores demonstrated different p53 expression levels. Over-expression of p53 protein was observed in 27/50 (54%) cases, whereas the rest of them (23/50-/46%) demonstrated moderate to low levels of the protein. P53 over-expression was statistically significantly correlated with the mitotic index of the examined cases (p=0.001). Interestingly, atypical/anaplastic group of histotypes demonstrated the strongest p53 expression rates compared to all others (p=0.001). No statistical significance was obtained when correlating p53 to the sex of the examined patients (p=0.981) or grade of differentiation (p=0.091). p53 high staining intensity values were observed in cases characterized by significantly high concentrations of stained nuclei.

Discussion

p53 alterations are observed in significant frequencies in solid malignancies affecting critically the proliferation/ apoptosis equilibrium (14). Mutations (substitutions, insertions/ deletions), and also epigenetic changes (aberrant gene

promoter methylation) are the main mechanisms of p53 dysfunction (15). Over-expression at the protein level is detected by implementing specific IHC assays. Concerning meningiomas, a difference in p53 expression levels seems to be a useful protein marker for the optimal stratification of the corresponding tissues regarding phenotype aggressiveness.

It is well known that meningiomas' histological origin is derived from arachnoid cap cells of the meninges in the brain periphery. Meningiomas include a broad spectrum of histo-sub-types (transitional, meningotheliomatous, fibrous, psammomatus, angiomatous, anaplastic, and atypical) (16). The most crucial histopathological sign correlated to an aggressive biological behavior is brain tissue invasion. Additionally, in the majority of meningiomas, extra-cranial metastatic potential is limited, whereas in some dedifferentiated types -including atypical and anaplastic- is increased (17). In these aggressive phenotypes, genetic analyses have already confirmed the presence of gross chromosomal and also specific gene imbalances (gains, rearrangements and intra- or inter- translocations, frame-shift deletions/insertions, in-frame fusions or point-driver mutations) (18-20). Numerical imbalances in chromosomes including chromosome 22, fragment deletions on chromosome 1p and 2q33-q35, specific regional chromosome 6p21-p22, 13q33, 17 and 19 amplifications have been also detected and reported (21-25). Furthermore, a variety of single nucleotide polymorphisms have been also recognized (26).

Our purpose in this experimental study was to explore the role of p53 protein expression in meningiomas by implementing an IHC-based protocol on tissue microarray cores. A broad spectrum of meningiomas' histological sub-types and grades was used as substrate for simultaneous analysis. Besides this, an objective, accurate method for staining intensity levels measurement was applied. According to our results, p53 expression was observed in high, moderate, and low levels in the corresponding examined tissues. Strong over-expression was associated with mitotic index. Interestingly, atypical/anaplastic group of histotypes demonstrated the strongest p53 expression rates. p53 over-expression -especially combined to ki67, a reliable cell proliferation marker- seem to be correlated to an increased tendency for recurrence in meningiomas of intracranial or intraspinal anatomical origin (27). Besides this, another study reported high p53 positive expression rates in meningiomas of the left frontal regions and parasellar-cavernous sinus, whereas ki67 over-expression was detected in the left frontal and bilateral parietal convexity (28). In contrast, another study concluded that although p53 and ki67 are over-expressed in meningiomas of Grade I, their recurrence risk is associated only with ki67 elevated indexes (29). Additionally, other protein expression analyses reported a significant relation between co-expression of the two markers with aggressive meningiomas' phenotypes including atypical histotypes (30-32). Interestingly, p53 indirect involvement in signaling transduction pathways such as PI3K/Akt, WNT, and hedgehog and in metabolic procedures -protein oxidation and lipid peroxidation- affects meningiomas' microenvironment. Especially, p53 mutant protein is associated with decreased antioxidant capacity in meningiomas (33, 34).

In conclusion, p53 over-expression affects negatively meningiomas' phenotypes; it correlates with elevated mitotic index and the aggressive atypical/anaplastic histotype). p53 over-expression demonstrates differences in meningiomas. Therefore, p53 could be a target for potential novel therapeutic strategies in meningiomas based on agents that could enhance p53-mediated apoptotic balance and response rates to specific chemo-radiation regimens.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

Roukas D, Tsiambas E: design of the study, manuscript writing; Kavantzas N, Lazaris AC, Kouzoupis A, Ragos V, Peschos D, Spyropoulou D: academic advisors; Tsouvelas G, Papanastasiou G, Falidas E, Manaios L, Papouliakos S, Manoli A, Katsinis S: collection and management of data, analysis/data interpretation. All Authors read and approved the final manuscript.

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