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Caspase 3 Expression Profiles in Meningioma Subtypes Based on Tissue Microarray Analysis

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Abstract. *Background/Aim: Concerning primary central nervous system neoplasms, meningiomas demonstrate the most common type in adults worldwide. Deregulation of apoptotic pathways in malignancies, including meningiomas, is correlated with chemoresistance and poor prognosis. Caspases represent crucial proteins that induce cell apoptosis. This study aimed to correlate caspase 3 protein expression levels to meningioma clinic-pathological features. Materials and Methods: A set of fifty (n=50) meningioma lesions was included in the current analysis including a broad spectrum of histopathological subtypes (meningotheliomatous, psammomatus, transitional, fibrous, angiomatous, microcystic, atypical and anaplastic). Immunohistochemistry was implemented on tissue microarray cores of selected paraffin blocks by applying an anti-caspase 3 antibody. Additionally, an image analysis protocol was also performed in the corresponding immunostained slides. Results: Caspase 3 protein over-expression was detected in 17/50 (34%) cases, whereas the remaining 33 cases (66%) were characterized by medium to low levels of the molecule. Caspase 3 expression was statistically significantly associated with the grade of the analyzed tumors and the mitotic index (p=0.002, p=0.001, respectively). Caspase 3 expression status was also correlated with the histotype of the selected meningiomas (p=0.016). Conclusion: Caspase 3 demonstrated low expression levels in a significant subset of the examined meningiomas correlated with differentiation grade, mitotic activity, and partially with specific histotypes. Agents that could enhance caspase 3 expression – inducing its apoptotic activity – represent a very promising area in oncology for developing novel treatment regimens.*

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Meningioma represents the second most frequent brain tumor derived from the intracranial primary central nervous system (CNS) in adults. Higher grade meningiomas are characterized by increased recurrence rates demonstrating an aggressive biological behavior that affects the response rates to surgery/radiation applied therapeutic regimens (1). The arachnoid cap cells of the meninges on the periphery of the brain represent the histological substrate for the onset of meningiomas. Meningotheliomatous, psammomatus, transitional, fibrous, angiomatous, atypical, and anaplastic represent distinct histopathological entities of meningiomas characterized by specific cytogenetical features (2). Brain tissue invasion is the most obvious histopathological finding

that reflects an aggressive biological behavior of the tumor. Additionally, meningiomas present a low level of extra-cranial metastatic potential. In fact, their metastatic activity and tissue to tissue penetration are extremely rare events. Molecular studies based on the analysis of large series of meningiomas have already reported a broad spectrum of gross chromosomal and specific gene modifications (rearrangements/intra- or inter- translocations, gains, frame-shift deletions/insertions, point-driver mutations or in-frame fusions) that influence the grade of differentiation (Grade I-III) in them (3-6). In conjunction to chromosome 22 numerical imbalances, fragment deletions have been detected on chromosome 1p and 2q33-q35. Additionally, multi-regional amplifications occur on chromosome 6p21-p22 and also on chromosomal 13q33, 17, and 19. Besides the chromosomal and gene alterations described above, meningiomas demonstrate a high level of somatic single nucleotide variants that appear as specific single nucleotide polymorphisms (SNPs) (7). Interestingly, there is limited evidence of viral implication in the onset, development, and progression of meningiomas. The main viral categories include human cytomegalovirus (HCMV), Epstein-Barr (EBV), HSV 6/7, human papilloma virus (HPV), and Hepatitis B virus (HBV) (8).

In cancerous tissues, apoptotic (programmed) cell death is desynchronized by alterations in pro- and anti-apoptotic proteins. This functional abnormality drives the cancer cells to immortalization, inducing tissue proliferation. Based on these imbalances, caspases and other apoptosis-related molecules, whether mitochondria-dependent or not, are considered important targets for specific therapeutic strategies aimed at enhancing the apoptotic levels of tumor cells (9, 10). In our original research study, we explored the role of caspase 3 (gene locus: 4q35.1) -a cysteine-aspartic acid protease involved in the execution stage of apoptosis- in meningiomas and its potential impact on meningioma pathological features using a tissue microarray digital image analysis assay.

Materials and Methods

Study group. A set of fifty (n=50) archival, formalin-fixed, and paraffin-embedded meningioma tissue specimens was selected. According to pathology classification histotypes, we identified 12 meningotheliomatous, 12 psammomatus, 6 transitional, 5 fibrous, 2 angiomatous, 2 microcystic, 5 atypical, 5 anaplastic, and 1 papillary meningioma. Concerning the corresponding patients, 39 (78%) were female, and 11 (22%) male. The ethics committee consented to the use of these tissues in the 1st Department of Pathology, Medical School University of Athens for research purposes, according to the World Medical Association Declaration of Helsinki. The extracted tissue sections were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides were evaluated by two independent pathologists for the final confirmation of histopathological diagnoses, including grading and p-staging. All neoplasms were classified according to the histological typing, grading, and mitotic index (mitoses per high power fields-HPF) criteria described in the World Health Organization (WHO) classification of tumors of the central nervous system guidelines (11, 12).

Tissue microarray (TMA) construction. Specific tissue spots characterized by elevated cancer cell concentration, were detected on the H&E-stained slides using a bright field microscope (Olympus BX- 50, Olympus Inc, Melville, NY, USA). TMA construction was based on the selection and tubal microsection of the corresponding selected blocks. The TMA construction assay was performed as described in our previous analysis by using the TMArrayer- 100 device (Chemicon International, Tamecula, CA, USA) (13). 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-caspase 3 antibody (monoclonal, clone 3CSP03-Neomarkers/LabVision, Fremont, CA, USA) at a 1:50 dilution was applied in the corresponding tissue tubal microsections. The IHC assay was performed as we described previously (13). Cytoplasmic and sub-membranous staining patterns were considered acceptable for caspase 3 expression profile (Figure 1B).

Digital image analysis assay (DIA). Caspase 3 protein expression levels were evaluated quantitatively by calculating the corresponding staining intensity levels (densitometry evaluation) in the stained cells (malignant). We implemented a DIA similarly with our previous analysis (13) (Figure 1C). Total results and DIA values are demonstrated in Table I.

Statistical analysis. The statistics software package IBM SPSS v25 (SPSS Inc, Chicago, IL, USA) was used for the analysis. The chi-square test (χ^2) for linear trend and Fisher's exact test were applied. Statistical significance (p) was evaluated in pairs and differences with $p<0.05$ were considered statistically significant. IHC results and differences (p-values) are described in Table I.

Results

According to the extracted DIA expression results, the meningioma tissue microarray cores exhibited varying levels of caspase 3 expression as determined by IHC. Specifically, strong caspase 3 protein expression (high staining intensity levels) was measured in 17/50 (34%) cases, whereas the remaining cases (33/50/66%) showed medium to low levels of immunoreactivity. Caspase 3 overall expression was found to be statistically significantly associated with the grade of the analyzed tumors and the mitotic index (p=0.002, p=0.001, respectively). Another critical observation was that caspase 3 expression status was associated with the histotype of the examined meningiomas (p=0.016). Specifically, caspase 3 expression demonstrated significant variations among histotypes. Papillary, microcystic, meningoepithelial, angiomatus, transitional, psammomatus, and fibrous meningiomas, exhibited different levels of caspase 3 expression, whereas anaplastic and atypical histotypes demonstrated distinct patterns (p=0.005). When correlating

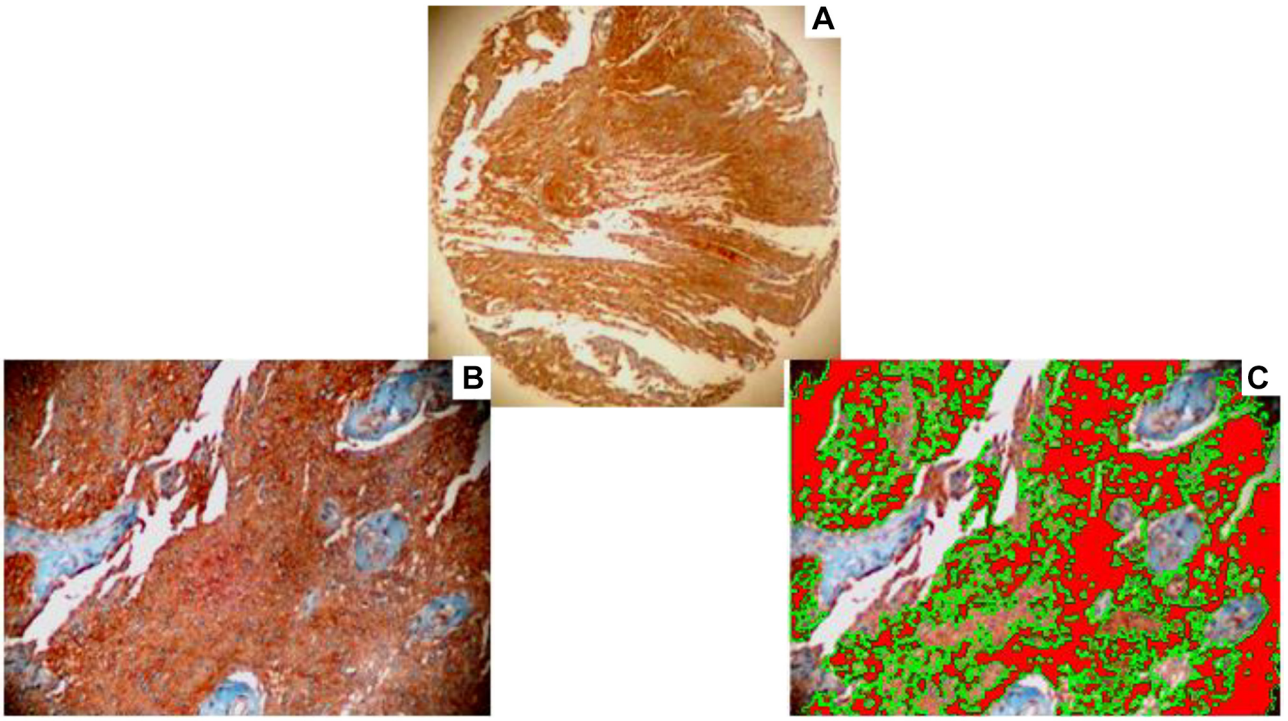

Figure 1. *High caspase 3 expression in a case of meningioma (transitional histotype). A) Tissue core immunostained by caspase 3 (original magnification 40×). B) Caspase 3 diffuse cytoplasmic and sub-membranous staining pattern (original magnification 100×). C) Digitized evaluation of caspase 3. Red/green areas represent different levels of protein expression corresponding to specific staining intensity levels across a spectrum of continuous grey scale RGB values ranging from 0 to 255.*

overall caspase 3 expression with patient sex, no statistical significance was found (p=0.382).

Discussion

Apoptosis is the precise term for characterizing the genetically programmed cell death orchestrated by specific molecular interactions. According to their origin and function, apoptotic proteins are involved in pathways that either induce or inhibit apoptosis (14). Two prominent and distinct apoptotic mechanisms are recognized: the extrinsic and intrinsic pathways. Various molecules act as inhibitors or inducers in this apoptotic process. Inducers include crucial proteins derived from the mitochondria. Cytochrome c, a critical molecule located in the inter-membrane space of the organelle, plays a pivotal role. Its release into the cytoplasm enhances activation of caspases (especially caspase-9) regulated by p53 and B-cell lymphoma-2 (Bcl-2) proteins (15). Caspases act as central promoters of apoptotic cell death. Biochemically, caspases are cysteine-aspartic proteases that regulate a wide range of cellular processes. More specifically, they critically influence cell homeostasis, inflammation, pyroptosis (a distinct aspect of programmed cell death mediated by microbial infection and immune response) necroptosis, tissue differentiation, and early embryonic development (16). Additionally, caspases function as tumor suppressors and are increasingly studied in relation to aging. By now, fifteen proteases have been isolated and cloned located on chromosomes 1, 2, 4, 7, 10, 11, 16, and 19. Initially inactive (pro-caspases), they become active through dimerization or oligomerization and subsequent cleavage, forming active heterotetrametric domains consisting of small and large subunits Based on their roles in apoptotic pathways, caspases are discerned as initiators (caspase-2,-8,-9, and -10) or executioners (caspase-3, -6, and -7) (17).

In order to fulfil the current research protocol, we analyzed a set of meningioma tissue cores (TMA) using IHC, selecting samples that represented various pathological forms and grades. Caspase 3 expression levels were categorized as high, moderate, or low associated with their differentiation grade, mitotic activity and specific histotypes. Interestingly, in our previous protein expression analysis regarding the p53 apoptosis regulator molecule in meningiomas, we showed that similarly to caspase 3 deregulation, its over-expression correlates with aggressive biological phenotypes, especially in atypical and anaplastic subtypes with higher recurrence rates (13).

Table I. *Clinicopathological parameters and caspase 3 expression results.*

Clinicopathological parameters		Caspase 3		*p*-Value
Meningiomas (n=50)		OE	MLE	
	17/50 (34%)	33/50 (66%)		
	n (%)	n	n	
Sex				0.382
Male	11 (22%)	3/50 (6%)	8/50 (16%)	
Female	39 (78%)	14/50 (28%)	25/50 (50%)	
Mitotic index (HPF)				
0-4	33/50 (66%)	2/50 (4%)	31/50 (62%)	**0.001**
>4≥19	10/50 (20%)	8/50 (16%)	2/50 (4%)	
≥20	7/50 (14%)	7/50 (14%)	0/50 (0%)	
Grade				**0.002**
I	36 (72%)	7/50 (14%)	29/50 (58%)	
II	8 (16%)	5/50 (10%)	3/50 (6%)	
III	6 (12%)	5/50 (10%)	1/50 (2%)	
Histo-type				**0.016**
Atypical	5/50 (10%)			
Anaplastic	5/50 (10%)			
Papillary	1/50 (0.5%)			
Meningotheliomatous	12/50 (24%)	5/50 (10%)	4/50 (8%)	
Psammomatus	12/50 (24%)	13/50 (26%)	13/50 (26%)	
Transitional	6/50 (12%)	11/50 (22%)	4/50 (8%)	
Fibrous	5/50 (10%)			
Angiomatous	2/50(4%)	21/50 (42%)	17/50 (34%)	
Microcystic	2/50(4%)	8/50 (16%)	4/50 (8%)	

OE: Over-expression (high expression) staining intensity values ≤130 at stained cells; MLE: moderate-low expression staining intensity values >130 at ≤160 at stained cells; bold values: statistically significant.

Other protein and gene expression analyses have focused on a variety of apoptotic factors including c-FLIP, XIAP, Bcl-2, caspase 3, 8, and 9, cytochrome c, APAF 1 and Smac/DIABLO, and reported low expression of caspase family proteins. The researchers revealed that c-FLIP inhibited these apoptotic inducers (18). Furthermore, over-expression of the tumor necrosis factor-related apoptosis-inducing ligand R2 (TRAIL-R2) in conjunction with caspase 8 down-regulation is a critical event frequently observed in meningiomas (19). In contrast to caspase 8, caspase 3 seems to be more frequently over-activated in meningiomas along with up-regulation of calpain (20). It is also important to be mentioned that survivin, a major apoptotic factor binding to caspases-3/7 external domains, is overactivated in meningiomas, inhibiting their function (21). Additionally, Midkine, a heparin-binding growth factor, interacts with caspase 3, inhibiting its activation and potentially affecting response rates to apoptotic cell death in meningioma cells *in vitro*, contributing to resistance against chemotherapeutic agents (22). Pharmacogenomic studies have focused on novel agents that induce apoptosis with fenretinide showing promising results. Fenretinide is a synthetic retinoid promoting apoptosis in malignant cell cultures across several malignancies. Research has demonstrated its ability to induce caspase activation in meningiomas. Furthermore, fenretinide induces apoptosis in meningiomas independently of their grade in a series of cancerous cell cultures (23). Moreover, valproic acid (VPA), a common anti-epileptic drug, seems to be implicated in apoptosis by up-regulating the expression of cleaved caspase-3 and PARP apoptotic molecules in meningioma stem cell cultures. Additionally, VPA induces radio-sensitivity rates in meningiomas (24). Recently, a novel target for specific treatment in meningiomas has been under investigation. A study group has focused on the impact of neurofibromatosis 2 (*NF2*) tumor suppressor gene. Schwannomas and meningiomas NF2-dependent malignancies demonstrate high levels of chemo-resistance due to mutations and gene modifications in signal transduction pathway inhibitors. They suggested that proteasomal pathway inhibitors in meningioma could be a very promising novel treatment strategy for cases characterized by gene alterations in *NF2* (25). In addition to these agents, a novel anti-apoptotic BCL-2 inhibitor, navitoclax, has demonstrated strong activity in malignant cell cultures by increasing the caspase 3 apoptotic function in combination with other targeted factors, such as everolimus and gemcitabine (26). Concerning

the impact of gemcitabine on meningioma therapeutic strategies, a study group showed that the agent blocked the progression of tumor cell proliferation by inducing apoptotic proteins, especially in high-grade cases (27).

Besides altered Caspase 3, brain tumors -including gliomas and high grade meningiomas – demonstrate Caspase 8 specific gene modifications. A genetic analysis observed that Caspase 8 D302H polymorphism is implicated crucially in their pathogenesis (28). In contrast, another study group concluded that Caspase 9 gene Ex5+32 G>A (rs1052576) polymorphism is correlated with a decreased risk for glioma brain tumor onset, acting as a protecting genetic factor in the corresponding carriers (29).

In conclusion, Caspase 3 protein expression varies among subsets of meningiomas, correlating with differentiation grade, mitotic activity, and specific histotypes in the examined cases. Caspase 3 is a major executioner in the apoptotic process, making it a very promising target for both single and combined therapeutic regimens in meningiomas. Concerning its role as an important and reliable predictive marker – such as p53 and ki67- there are very limited data (30). Enhancement of its activity by novel agents could potentially increase caspase-mediated apoptotic death and improve treatment response rates in patients characterized by specific protein and gene signatures receiving chemo-radiation regimens (31).

Conflicts of Interest

The Authors have no financial or non-financial interests to disclose.

Authors' Contributions

All Authors contributed to the study conception and design. DR, ET, SM, A-EM: Materials preparation, data collection and analysis, DR, ET: Draft writing, Draft reviewing, academic advisors: DS, MA, GT, AK, AL, NK. All Authors read and approved the final manuscript.

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